

US009353401B2

(12) **United States Patent**  
**Walia**

(10) **Patent No.:** **US 9,353,401 B2**  
(45) **Date of Patent:** **May 31, 2016**

(54) **MULTIPLEX ASSAYS WITH MULTIPLE LUCIFERASES REPORTERS AND USES THEREOF**

(75) Inventor: **Rampyari Walia**, Alpine, CA (US)  
(73) Assignee: **Targeting Systems**, El Cajon, CA (US)  
(\* ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 43 days.

(21) Appl. No.: **13/393,170**

(22) PCT Filed: **Aug. 27, 2010**

(86) PCT No.: **PCT/US2010/047033**

§ 371 (c)(1),  
(2), (4) Date: **Feb. 28, 2012**

(87) PCT Pub. No.: **WO2011/025980**

PCT Pub. Date: **Mar. 3, 2011**

(65) **Prior Publication Data**  
US 2012/0156705 A1 Jun. 21, 2012

**Related U.S. Application Data**

(60) Provisional application No. 61/238,146, filed on Aug. 29, 2009.

(51) **Int. Cl.**  
*C12Q 1/66* (2006.01)  
*C12N 9/02* (2006.01)

(52) **U.S. Cl.**  
CPC ..... *C12Q 1/66* (2013.01); *C12N 9/0069* (2013.01)

(58) **Field of Classification Search**  
USPC ..... 435/8  
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,604,123	A *	2/1997	Kazami et al. ....	435/189
6,495,355	B1 *	12/2002	Contag et al. ....	435/189
7,723,502	B2 *	5/2010	Coleman et al. ....	536/23.1
8,367,357	B2 *	2/2013	Ohmiya et al. ....	435/8
2005/0037355	A1 *	2/2005	Day et al. ....	435/6
2005/0112551	A1 *	5/2005	Blair et al. ....	435/5
2005/0153310	A1 *	7/2005	Fan et al. ....	435/6
2008/0193956	A1 *	8/2008	Kricka et al. ....	435/8
2008/0274485	A1	11/2008	Walia	
2009/0081715	A1 *	3/2009	Burns-Guydish et al. ....	435/8
2009/0136998	A1 *	5/2009	Gambhir et al. ....	435/69.1
2010/0055693	A1 *	3/2010	Leu et al. ....	435/6
2010/0092967	A1 *	4/2010	Leu et al. ....	435/6

OTHER PUBLICATIONS

Bennett et al., "Development of a dual-luciferase fusion gene as a sensitive marker for site-directed DNA repair strategies", The Journal of Gene Medicine, vol. 5, pp. 723-732, 2003.\*  
GenBank Accession No. AAB86460.1, published Nov. 17, 1997.\*

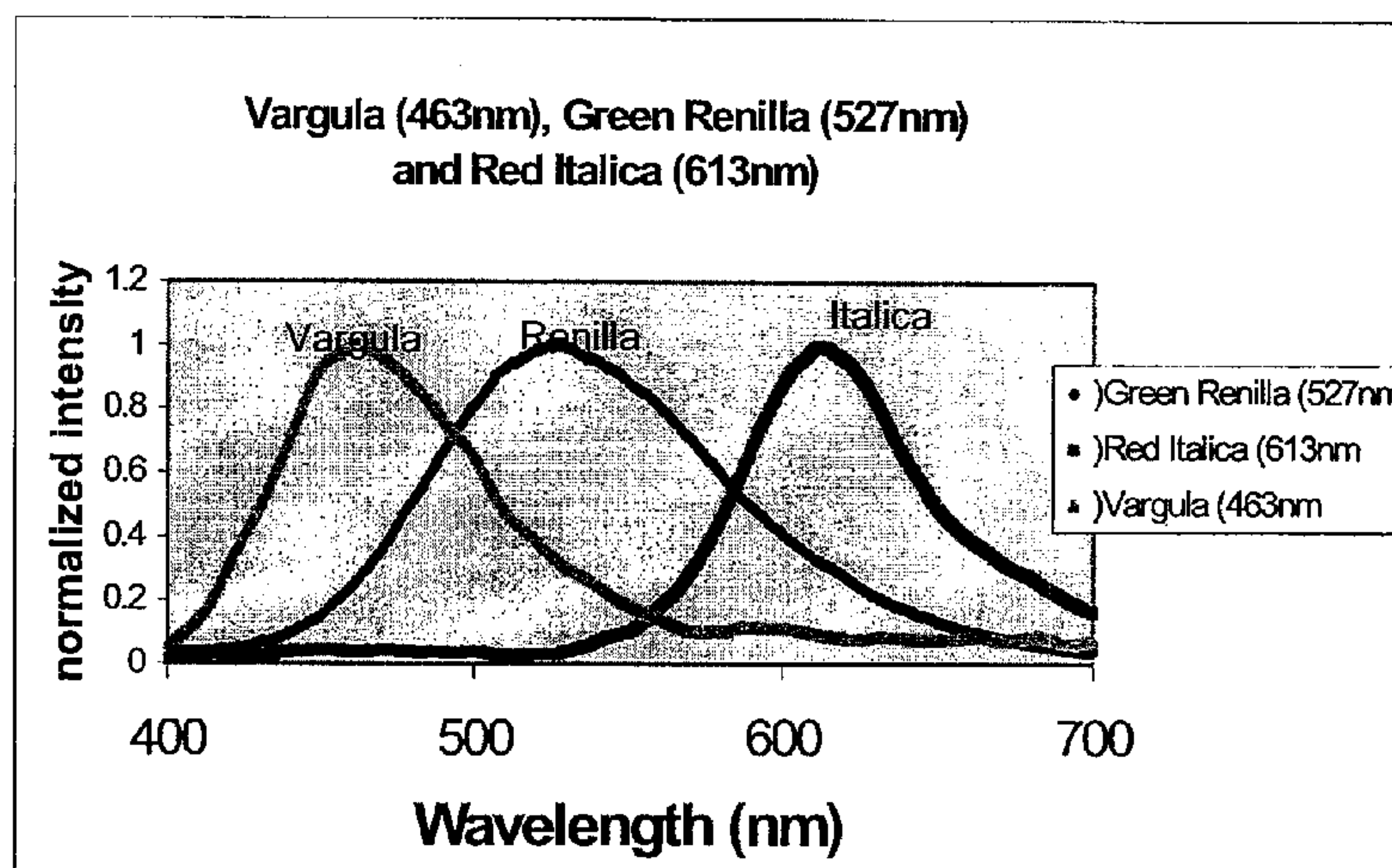
(Continued)

*Primary Examiner* — Robert Mondesi  
*Assistant Examiner* — Richard Ekstrom  
(74) *Attorney, Agent, or Firm* — Pepper Hamilton LLP

(57) **ABSTRACT**

The present invention encompasses modified luciferases, methods for making modified luciferases, and assays utilizing modified luciferases. Modified luciferases of the invention show increased activity over wildtype luciferases and also show increased stability of signal. The present invention also encompasses multiplex assays utilizing multiple luciferases reporters with different emission spectra and different substrates for simultaneous luciferase measurements.

**1 Claim, 42 Drawing Sheets**



(56)

**References Cited**

OTHER PUBLICATIONS

Branchini, B.R., et al., "Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications," *Anal. Biochem.* Feb. 15, 2007;361(2):253-262.

Kitayama et al., "An in vivo dual-reporter system of cyanobacteria using two railroad-worm luciferases with different color emission," *Plant Cell Physiology*, 2004, vol. 45, No. 1, pp. 109-113.

Michelini et al., "Spectral-Resolved Gene Technology for Multiplexed Bioluminescence and High-Content Screening," *Analytical Chemistry*, 2008, vol. 80, No. 1, pp. 260-267.

Stern et al., "Improving mammalian cell factories: The selection of signal peptide has a major impact on recombinant protein synthesis and secretion in mammalian cells," *Trends in Cell and Molecular Biology* (online), 2007, <http://unitargeting.com/Resources/Trends07.pdf>, p. 6, col. 2, para 5 to p. 7, col. 1, para 1; p. 7, col. 1, para 4; p. 8, col. 1, para 1; p. 9, col. 1, para 3; Table 1.

Wu, C., et al., "Dual-reporter assay using two secreted luciferase genes," *Biotechniques*. Mar. 2007;42(3):290-292.

Relevant Portion of International Search Report, PCT/US2010/047033, mailed Nov. 18, 2010.

\* cited by examiner



FIG. 1

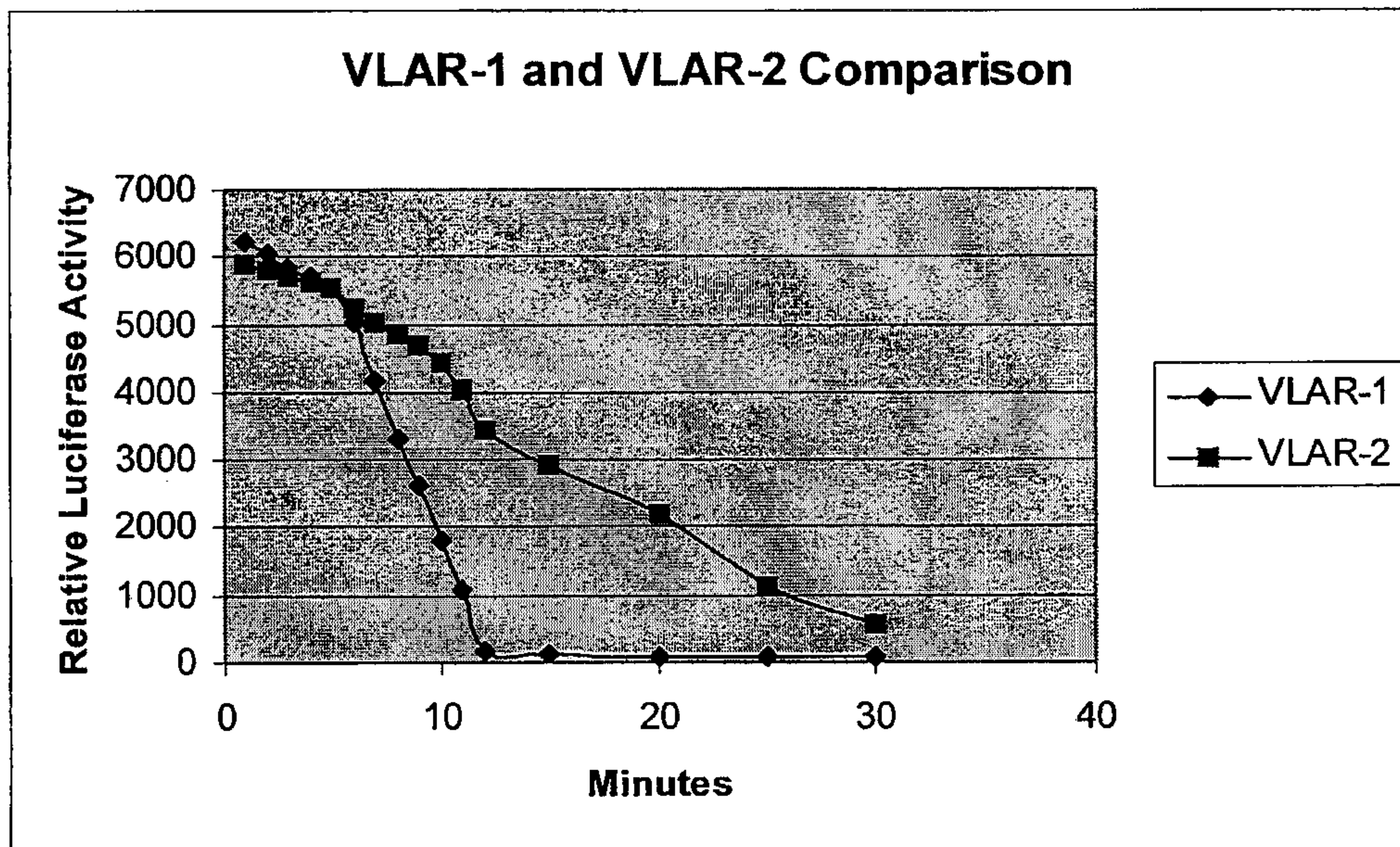


FIG. 2A. Sample volume 20  $\mu$ l

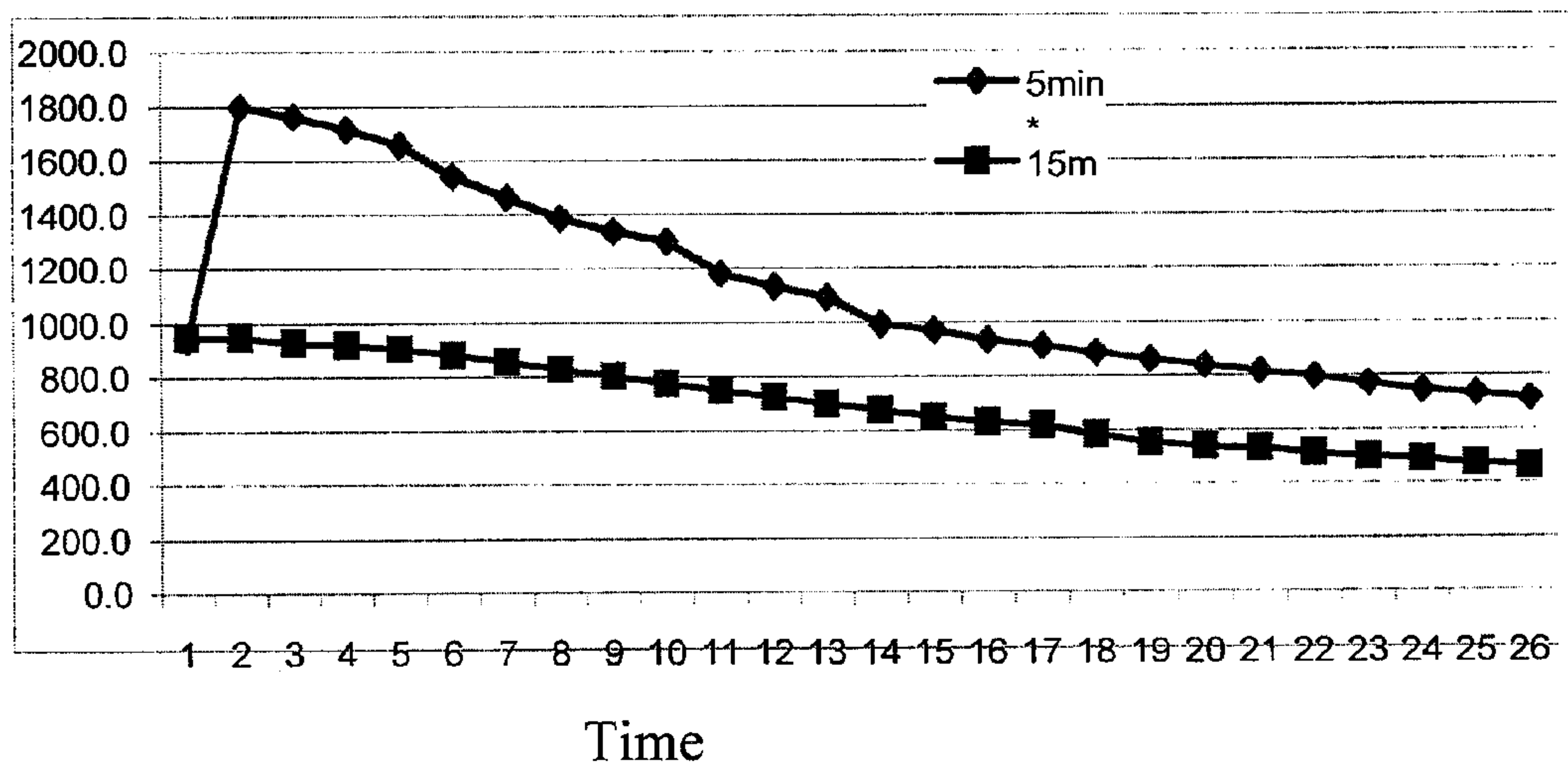


FIG. 2B. Sample volume 5

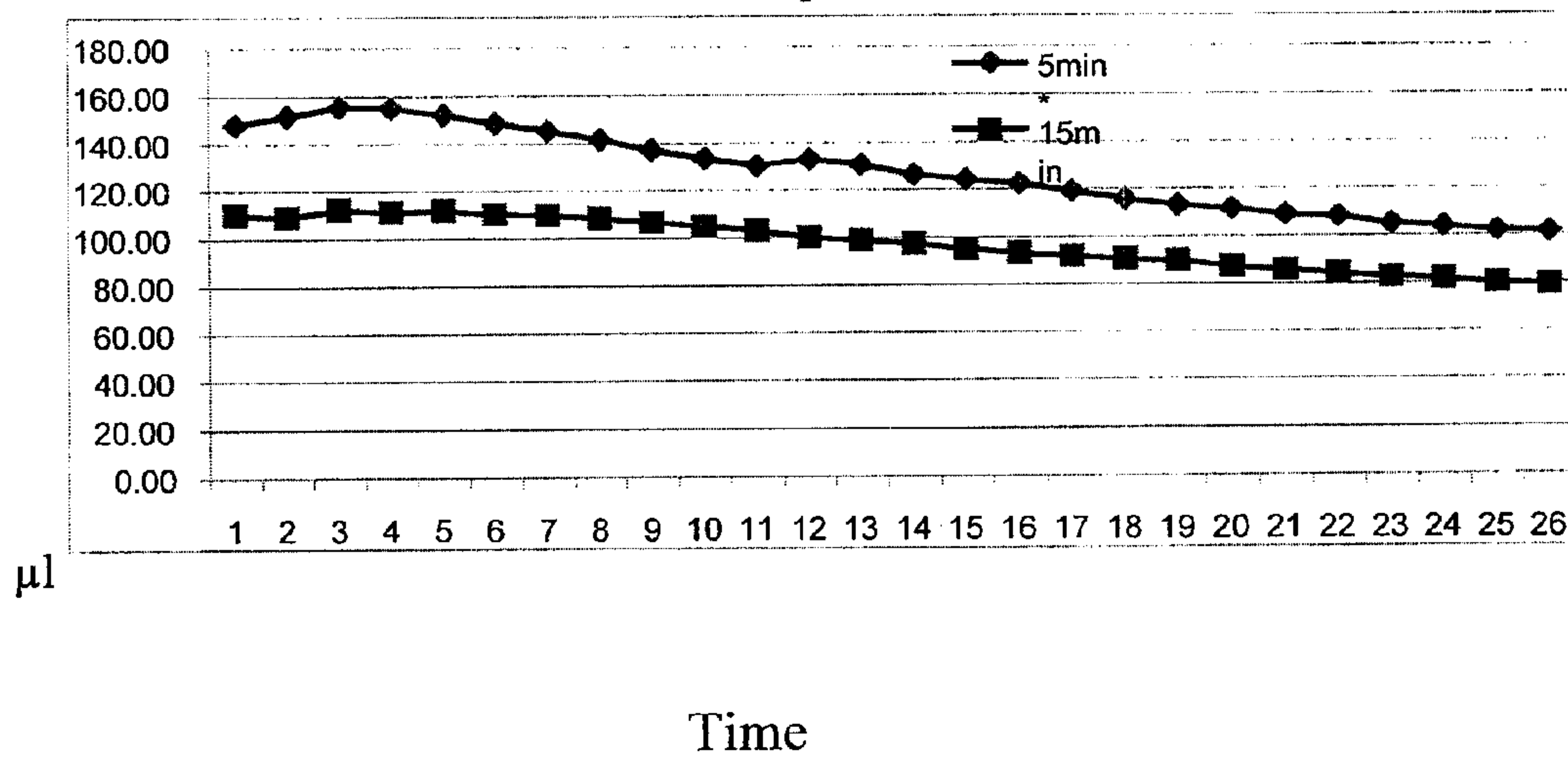




FIG. 3A

**pCMV Green Renilla Luciferase plasmid Sequence (SEQ ID NO: 1):**

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg  
61 ccgcatagtt aagccagtat ctgctccctg ctgtgtgtt ggaggctgct gagtagtgcg  
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc  
181 ttagggtag gcgtttgcg ctgctcgcg atgtacgggc cagatatacg cgttgacatt  
241 gattattgac tagtattaa tagtaataca ttacggggtc attagttcat agcccatata  
301 tggagtccg cgttacataa ctacggtaa atggcccgcg tggctgaccg cccaacgacc  
361 cccgcccatt gacgtcaata atgacgatg ttcccatagt aacgccaata gggactttcc  
421 attgacgtca atgggtggac tatttacggg aaactgcccc ctggcagta catcaagtgt  
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt  
541 atgcccagta catgacctta tgggacttcc ctacttggca gtacatctac gtattagtca  
601 tcgctattac catggtgatg cggtttggc agtacatcaa tgggcgtgga tagcggtttg  
661 actcacgggg attccaagt ctccacccca ttgacgtcaa tgggagtttg tttggcacc  
721 aaaatcaacg ggactttcca aaatgtcgt acaactcgc cccattgacg caaatgggcg  
781 gtaggcgtgt acgggtggag gtctatataa gcagagctct ctggctaact agagaacca  
841 ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gcttggtagc  
901 gagctcggat ccatgtgtt gaaagtgtg tttgctattg gatgtatcgt agtgcaggct  
961 atggcctcaa aagtgtacga tccggagcag cggaagagga tgatcacggg gcccattgg  
1021 tgggcacgat gcaagcagat gaatgtgtg gacagttca ttaactacta cgacagcgag  
1081 aaacacgcgg agaacgcagt gatattcctg cacggcaatg caaccagtag ctatctgtg  
1141 agacacgtgg tgccicatat tgagccggtc gctagatgca ttattcccga tcttattgga  
1201 atgggaaat cgggaaagag tggaaatgga tcatatagcc tctcogatca ttataaatat  
1261 ctgactgctt ggttgaatt gctcaatctg cccaagaaaa tcatcttgt aggacatgat  
1321 tggggctccg ccctgctt tcaattatgcc tatgaacacc aggatcggat caaggctatt  
1381 gttcacatgg agagcgtgtt ggatgtgatt gaatcatgga tgggtggcc ggatatagaa  
1441 gaagagctgg cgctgattaa atctgaggag ggcgagaaga tggactcga aaataacttc  
1501 ttgtcgaga cggactgcc cagtaagatc atgcgcaaac tggagcctga agagttgcg  
1561 gcttacctgg aaccctcaa ggagaaggga gaggtgagga gaccgacct gcatggcct  
1621 cgggaaatc cgctggtaaa aggaggaag ccagacgtcg tcgccattgt ccggaattac  
1681 aacgcttacc tccgcgctag tgacgacctg cctaaactct tcatcgaatc agatcctgtt  
1741 ttcttagta acgcatcgt cgagggcgcc aagaagttc caaacaccga attgttaaa  
1801 gtcaaaggac ttacttctt ccaggaggat gcgcccgatg aaatgggaaa gtatatcaaa  
1861 tcctcgtgg agaggtctt gaagaatgag cagaggtcca tctagtctag aaataattct  
1921 tactgtcatg ccaagtaaga tgctttctg tgctgcaata gcaggcatgc tgggatgcg  
1981 gtgggctcta tggctctga ggcgaaaga accagctggg gctctagggg gtatccccac  
2041 gcgcccgtga gcggcgcat aagcgcggcg ggtgtgtgg ttacgcgcag cgtgaccgct  
2101 acactgcca gcgccctagc gcccgctct tctgcttct tcccttctt tctcgccacg  
2161 ttcgcccgtt tccccgtca agctctaaat cggggcatcc cttaggggt cggattagt  
2221 gctttacggc acctcgacc caaaaaact gattaggggt atggttcacg tagtgggcca  
2281 tcgcccgtat agacggttt tcgcccctt acgttggagt ccacgttct taatagtgga  
2341 ctctgttcc aaactggaac aacctcaac cctatctgg tctattctt tgattataa  
2401 gggatttgg ggattcggc ctattggta aaaaatgagc tgatttaaca aaaattaac  
2461 gcgaattaat tctgtggaat gtgtgtcagt taggtgtgg aaagtccca ggctccccag  
2521 gcaggcagaa gtatgcaag catgcatctc aatagtcag caaccagggtg tggaaagtcc  
2581 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata  
2641 gtcccggccc taactccgc catcccggcc ctaactcgc ccagttccgc ccattctccg  
2701 ccccatggct gactaattt tttatttat gcagaggccg aggcgcctc tgcctctgag  
2761 ctattccaga agtagtgagg aggttttt ggaggcctag gctttgcaa aaagctcccg  
2821 ggagctgta tatccattt cggatctgat caagagacag gatgaggatc gtttcgcatg  
2881 attgaacaag atggattgca cgcaggtct cggccgctt ggggtgagag gctattcggc  
2941 tatgactggg cacaacagac aatcggctgc tctgatgcc cgtgttccg gctgtcagcg  
3001 cagggcgcc cggttctt tgtcaagacc gacctgccc gtgccctgaa tgaactgcag  
3061 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc  
3121 gacgtgtca ctgaagcggg aaggactg ctgctattg gcgaagtgcc ggggcaggat



FIG. 3B

3181 ctctgtcat ctcacctgc tctgcccag aaagatcca tcatggctga tgcaatgcg  
3241 cggctgcata cgctgatcc ggctaccctgc ccattcgacc accaagcgaa acatcgcatc  
3301 gagcgagcac gtactcggat ggaagccggc ctgtcgcac aggatgatct ggacgaagag  
3361 catcaggggc tcgcccagc cgaactgtc gccaggctca aggcgcgcat gcccgacggc  
3421 gaggatctcg tcgtgacca tggcgatgcc tgcctgccga atatcatggt ggaaaatggc  
3481 cgctttctg gattcatcga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata  
3541 gcgttggcta cccgtgatat tgctgaagag ctggcggcg aatgggctga ccgcttcctc  
3601 gtgcttacg gtatcgccgc tcccattcg cagcgcacgc ccttctatcg ccttctgac  
3661 gagttctct gagcgggact ctggggctcg aatgaccga ccaagcgacg cccaacctgc  
3721 catcacgaga ttctgattcc accgcccct tctatgaaag gttgggctc ggaatcggtt  
3781 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttctcgccc  
3841 acccaactt gttattgca gcttataatg gttacaaata aagcaatagc atcaciaaatt  
3901 tcaciaaata agcattttt tctctgact ctagtgtgg ttgtccaaa tcatcaatg  
3961 tctctatca tctctgata ccgtcgacct ctactagag ctggcgtaa tcatggctat  
4021 agctgttcc tgtgtgaaat tcttatccgc tcacaattcc acacaacata cgagccggaa  
4081 gcataaagt taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc  
4141 gctcactgcc cgcttccag tcgggaaacc tctcgtgcca gctgcattaa tgaatcggcc  
4201 aacgcgcggg gagagggcgt ttgcgtattg ggcgctctc cgcttctcg tctactgact  
4261 cgctgcgctc ggtcgtcgg ctgcggcag cggtatcagc tactcaaag gcggaatac  
4321 ggatccac agaatcagg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa  
4381 agccaggaa ccgtaaaaag gccgcgttc tggcgtttt ccataggctc cccccctg  
4441 acgagcatca caaaaatcga cgctcaagtc agaggtggc aaaccgcaca ggactataaa  
4501 gataccaggc gttccccct ggaagctccc tctgctcgc tctgttccg accctgccgc  
4561 ttaccggata cctgtccgc tttctcctt cgggaagcgt ggcgcttct caatgctac  
4621 gctgtaggta tctcagttcg gtgtaggctg tctcctcaa gctgggctgt gtgcacgaac  
4681 cccccgtca gcccgaccgc tgcgcctat ccggaacta tctcttgag tccaaccgg  
4741 taagacacga ctatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt  
4801 atgtaggcgg tctacagag tcttgaagt ggtggcctaa ctacggctac actagaagga  
4861 cagtattgg tctcgcgt ctgctgaagc cagtacctt cggaaaaaga gttgtagct  
4921 ctgatccgg caaacaacc accgctggtg gcggtggtt tttgttgc aagcagcaga  
4981 ttacgcgag aaaaaagga tctcaagaag atcctttgat ctttctacg ggtctgacg  
5041 tctagtggaa cgaaaactca cgttaagga tttggtcat gagattatca aaaaggatct  
5101 tcacctagat cttttaaata taaaatgaa gtttaaata aatctaaagt atatagat  
5161 aaacttggc tgacagttac caatgctaa tcatgaggg acctatctca gcatctgtc  
5221 tattctgtc atccatagtt gctgactcc ccgtcgtgta gataactacg atacgggagg  
5281 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccgctccag  
5341 attatcagc aataaaccag ccagccggaa gggccgagcg cagaagtgg cctgcaactt  
5401 tatccgctc catccagctt attaatggt gccgggaagc tagagtaagt agttcgcag  
5461 ttaatagtt gcgcaacgtt gttgccattg ctacaggcat cgtggtgtca cgctcgtct  
5521 ttggtatggc tctatcagc tccggtccc aacgatcaag gcgagttaca tgatcccca  
5581 tgtgtgcaa aaaagcgggt agctcctcg gtcctccgat cgtgtcaga agtaagtgg  
5641 ccgcatggt atcactcatg gttatggcag cactgcataa tctcttact gcatgccat  
5701 ccgtaagatg ctttctgtg actggtgagt actcaaccaa gtcattctga gaatagtga  
5761 tgcggcacc gagttgctt tcccggcgt caatacggga taataccgcg ccacatagca  
5821 gaacttaaa agtgctcatc attgaaaac gttctcggg gcgaaaactc tcaaggatct  
5881 taccgctgt gagatccagt tcgatgtaac ccactcgtc acccaactga tctcagcat  
5941 ctttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaa  
6001 agggaataag ggcgacacgg aatgtgaa tactcact cttctttt caatattat  
6061 gaagcattta tcagggttat tctctatga gcgatacat attgaaatg attagaaaa  
6121 ataaacaat aggggtccg cgcacattc cccgaaaagt gccacctgac gtc



FIG. 4A

## Modified red firefly luciferase with secretory signal (SEQ ID NO: 2)

1 gacggatcgg gagatcctcc gatcccctat ggtcgactct cagtacaatc tgctctgatg  
61 ccgcatagtt aagccagtat ctgctccctg ctgtgtgtt ggaggctgct gagtagtgcg  
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc  
181 ttagggtag gcgtttgcg ctgctcgcg atgtacgggc cagatatacg cgttgacatt  
241 gattattgac tagtattaa tagtaatcaa ttacggggtc attagttcat agcccatata  
301 tggagtccg cgttacataa ctacggtaa atggcccgcg tggctgaccg cccaacgacc  
361 cccgccatt gacgtcaata atgacgatg ttccatagt aacgccaata gggactttcc  
421 attgacgtca atgggtggac tattacggg aaactgcca ctggcagta catcaagtgt  
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gctggcatt  
541 atgccagta catgacctta tgggactttc ctactggca gtacatctac gtattagta  
601 tcgctattac catggatgag cggtttggc agtacaatca tgggcgtgga tagcggttg  
661 actcacgggg atttccaagt ctccaccca ttgacgtcaa tgggagttg tttggcacc  
721 aaaatcaacg ggactttcca aaatgtcgt acaactccgc cccattgacg caaatgggcg  
781 gtaggcgtgt acgggtgggag gtctatataa gcagagctct ctggctaact agagaacca  
841 ctgctactg gcttatgaa ataatagca ctactatag ggagaccaa gcttggtacc  
901 gagctcggat cc atggccttccctgtggctgctgtcctgctgggcccctgctgggcaccaccttcggc  
961 taccgatcg aggagggtc tgcggcctc caattgcaca agtacaatgca acaatcggc  
1021 aagctcggcg ccatgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag  
1081 tacttcgaca tcacgtgcag actcggcag gctatgaaga actacggcat gaagccagaa  
1141 ggacacatcg ctctctgtag cgagaactgc gaagagtct tcaattctgt tctggctggt  
1201 cttacatcg gagttacgt cgcgccaact aacgaaatt atacactag agagctgaac  
1261 cacagtctgg ggatagcca acctactatc gtattctca gcaggaagg cctgccc aaa  
1321 gtgctgagg tgcagaagac cgtgacttgc atcaaaacca ttgtatcct ggacagtaag  
1381 gtcaactcg ggggtatga ctgcgtagag acctcatta agaaacacgt cgagctgggc  
1441 tttctgcca cctcattgt gccatcgc gtcaaagacc ggaagacca cttgctctg  
1501 cttatgaact ctccgggtc cacagggctg ccaaaggag tagagatcac tcacgaggcc  
1561 ctggtcacga gattctca cgtaaggac cctatatac gcaatcaggt ggcccaggt  
1621 accgctacc tgactgtgt gccttccac cacggctcg gaatgtcac tacttgggc  
1681 tacttgcct gcggtaccg gattgtcat ctactaagt tcgacgagga gctttcctg  
1741 cgcacactc aggattaca gtgcactaca gtaatcctgg tccgacact gttcgcaatt  
1801 ctaataggt ctgagctct tgataagtt gacctcta acctgactga aatagccagc  
1861 ggtggtgct cacttgcaa ggagatcggc gaggtgtg caagaagatt caacctcca  
1921 ggcgtccggc agggatatg actcaccgag actaccagtg ctttatcat cactcctaag  
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgtcaaggi gaagattatt  
2041 gacctgata ccaagaaaac gttgggtgc aacagacggg gagaaatctg cgtgaaagga  
2101 ccatctcta tgtgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag  
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca ctcttata  
2221 gtcgaccgcc tgaatctct cattaagtat aaaggatacc aagtgccacc agctgaactg  
2281 gactctgtc tctgcaaca ccctaacatt agagatgctg gtgtggccgg ggtcccgc  
2341 agcagggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact  
2401 gagaaagaaa tcgtagacta tgtaaactcc caggtgtca accacaagcg gctgaggggc  
2461 ggcgtcggg tcgtagatga agtcccaag ggcctcacag gaaagatcga cgcgaaagt  
2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctgatctag aaataattc  
2581 tactgtcatg ccaagtaaga tgccttctg tgctgcaata gcaggcatgc tggggatgcg  
2641 gtgggctca tggcttctga ggcggaaaga accagctggg gctctagggg gtatccccac  
2701 gcgcccgtg gcggcgcat aagcgcggcg ggtgtggtg ttacgcgcag cgtgaccgct  
2761 acactgcca gcgccctagc gcccgctct tgccttct tccctcct tctgcccag  
2821 ttgccggct ttccccgta agctctaaat cggggcatcc cttaggggt cagattagt  
2881 gcttacggc acctcgacc caaaaaact gattaggggt atggitcacg tagtgggcca  
2941 tgcctgat agacggttt tgccttgg acgtggagt ccacgtctt taatagtga  
3001 ctctgtcc aaactggaac aacctcaac cctatctcg tctattctt tgattataa  
3061 gggatttgg ggattcggc ctattggtt aaaaatgagc tgatttaaca aaaattaac  
3121 gcgaattaat tctgtggaat gtgtgtagt tagggtgtg aaagtccca ggctcccag  
3181 gcaggcagaa gtatgcaaag catgcatctc aatagtcag caaccagggt tggaaagtcc



FIG. 4B

3241 ccaggctccc cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata  
3301 gtcccgcccc taactccgcc catcccgccc ctaactccgc ccagttccgc ccattctccg  
3361 ccccatggct gactaatttt ttttaattat gcagaggccg aggccgcctc tgcctctgag  
3421 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttgcaa aaagctcccc  
3481 ggagcttcta tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg  
3541 attgaacaag atggattgca cgcaggttct ccggccgctt gggtgagag gctattcggc  
3601 taigactggg cacaacagac aatcggctgc tctgatgccg ccgtgtccg gctgtcagcg  
3661 cagggcgcc cggttcttt tgtcaagacc gacctgtccg gtgccctgaa tgaactgcag  
3721 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttcttgccg agctgtgctc  
3781 gacgttgca ctgaagcggg aaggactgg ctgctattgg gcgaagtgc ggggcaggat  
3841 ctctgtcat ctacctgc tctgccgag aaagtatcca tcatggctga tgcaatgcgg  
3901 cggctgcata cgcttgatcc ggctacctgc ccattcgacc accaagcgaac acatgcac  
3961 gagcgagcac gtactcggat ggaagccggt ctgtcgcac aggatgatct ggacgaagag  
4021 catcaggggc tcgcccagc cgaactgtc gccaggctca aggcgcgcac gcccgacggc  
4081 gaggatctcg tcgtgaccca tggcgatgcc tgctgccga atatcatggt gaaaatggc  
4141 cgctttctg gattcatga ctgtggccgg ctgggtgtg cggaccgcta tcaggacata  
4201 gcgttgcta cccgtgatat tctgaagag ctggcggcg aatgggctga ccgcttctc  
4261 gtgcttacg gtatgccgc tcccattcg cagcgatcg cctctatcg ccttctgac  
4321 gagtctct gagcgggact ctggggtcg aatgaccga ccaagcgacg cccaacctgc  
4381 catcacgaga ttctgattcc accgccgct tctatgaaag gttgggctc ggaatcgtt  
4441 tccgggacgc cggctggatg atctccagc gcggggatct catgctggag ttctcggcc  
4501 acccaactt gttattgca gcttataatg gttacaaata aagcaatagc atcacaatt  
4561 tcacaaataa agcattttt tcaactgact ctagtgtgg ttgtccaaa ctcatcaatg  
4621 tatcttaca tctctgata ccgtcgacct ctactagag ctggcgtaa tcatggctat  
4681 agctgttcc tgtgtgaaat tttatccgc tcacaattcc acacaacata cgagccgga  
4741 gcataaagt taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttc  
4801 gctcactgcc cgcttccag tcgggaaacc tgcgtgcca gctgcatta tgaatcggc  
4861 aacgcgcggg gagaggcgt ttgcgtattg ggcgctctc cgcttctcg ctactgact  
4921 cgctgcgctc ggtcgttcgg ctgcggcgag cggatcagc tcaactaaag gcgtaatac  
4981 gttatccac agaactcagg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa  
5041 agccaggaa ccgtaaaaag gccgcgttc tggcgtttt ccataggctc cgccccctg  
5101 acgagcatca caaaaatcga cgctcaagtc agaggtggcg aaaccgaca ggactataaa  
5161 gataccaggc gttccccct ggaagctccc tcgtgcgctc tctgttccg acctgccc  
5221 ttaccgata cctgtccgc ttctccctt cggaagcgt ggcgcttct caatgctac  
5281 gctgtaggta tctcagttcg gtaggttcg ttgcctcaa gctgggctgt gtgcacgaac  
5341 ccccgctca gccgaccgc tgcgcttat ccgtaacta tctcttgag tccaaccgg  
5401 taagacagc cttatgcca ctggcagcag ccactgtaa caggattagc agagcgagg  
5461 atgtaggcgg tctacagag ttctgaagt gttggcctaa ctacggctac actagaagga  
5521 cagtattgg taactgcgct ctgctgaagc cagtacctt cggaaaaaga gttgtagct  
5581 ctgatccgg caaacaacc accgctggtg gcggtggtt tttgtttgc aagcagcaga  
5641 ttacgcgcag aaaaaagga tctcaagaag atcctttgat ctttctacg ggtctgacg  
5701 ctactggaa cgaaaactca cgttaagga tttggtcat gagattatca aaaaggatct  
5761 tcacctagat cttttaaata taaaaatgaa gttttaaata aatctaaagt atatatgagt  
5821 aaactggtc tgacagtac caatgctaa tcaagtggc acctatcga cgcactgtc  
5881 tattcgttc atccatagt gctgactcc ccgtcgtgta gataactac atacgggagg  
5941 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag  
6001 attatcagc aataaaccag ccagccgga gggccgagcg cagaagtgg cctgcaact  
6061 tatccgctc catccagtct ataatgtt gccgggaagc tagagtaagt agttcggcag  
6121 ttaatagtt gcgcaacgtt gttgccattg ctacaggcat cgtggtgca cgctcgtct  
6181 ttggtatggc ttactcagc tccggtccc aacgatcaag gcgagtaca tgatcccca  
6241 tgttgcaaa aaaagcgtt agctccttcg gtcctccgat cgtgtcaga agtaagtgg  
6301 ccgactggt atcactcatg gttatggcag cactgcataa ttcttact gtcatgcat  
6361 ccgtaagatg ctttctgtg actggtgagt actcaacca gtcattctga gaatagtga  
6421 tgcggcagc gagtgcctc tcccggcgt caatacggga taatacggc ccacatagca  
6481 gaactttaa agtgctcatc atggaaaac gttctcggg gcgaaaactc tcaaggatct  
6541 taccgctgt gagatccagt tcgatgtaac ccactcgtc acccaactga tctcagcat



**FIG. 4C**

6601 ctttacttt caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa  
6661 agggaataag ggcgacacgg aaatgtgaa tactcact cttcctttt caatattatt  
6721 gaagcatta tcagggttat tgtctcatga gcggatacat attgaaatgt attagaaaa  
6781 ataaacaaat aggggttccg cgcacattc cccgaaaagt gccacctgac gtc

FIG. 5

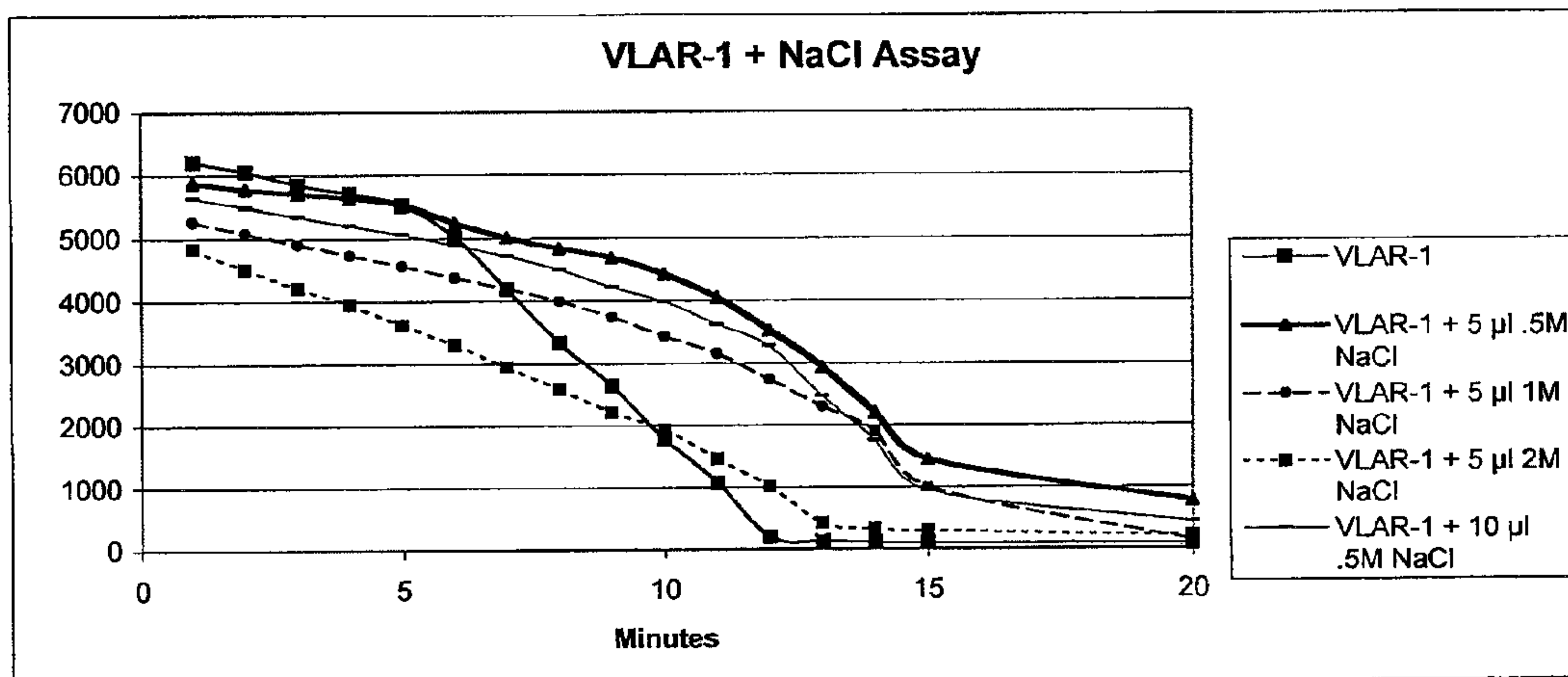




FIG. 6

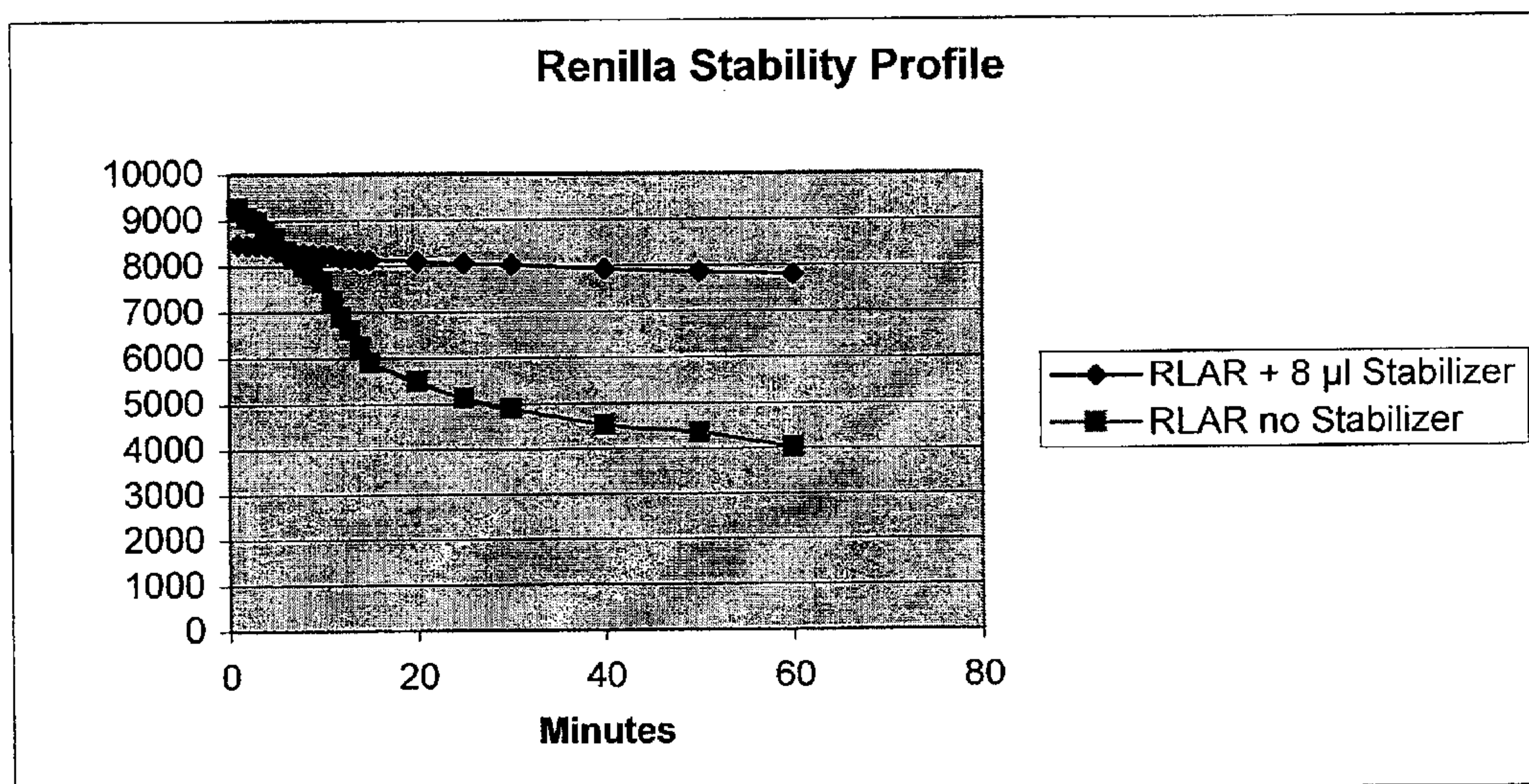


FIG. 7

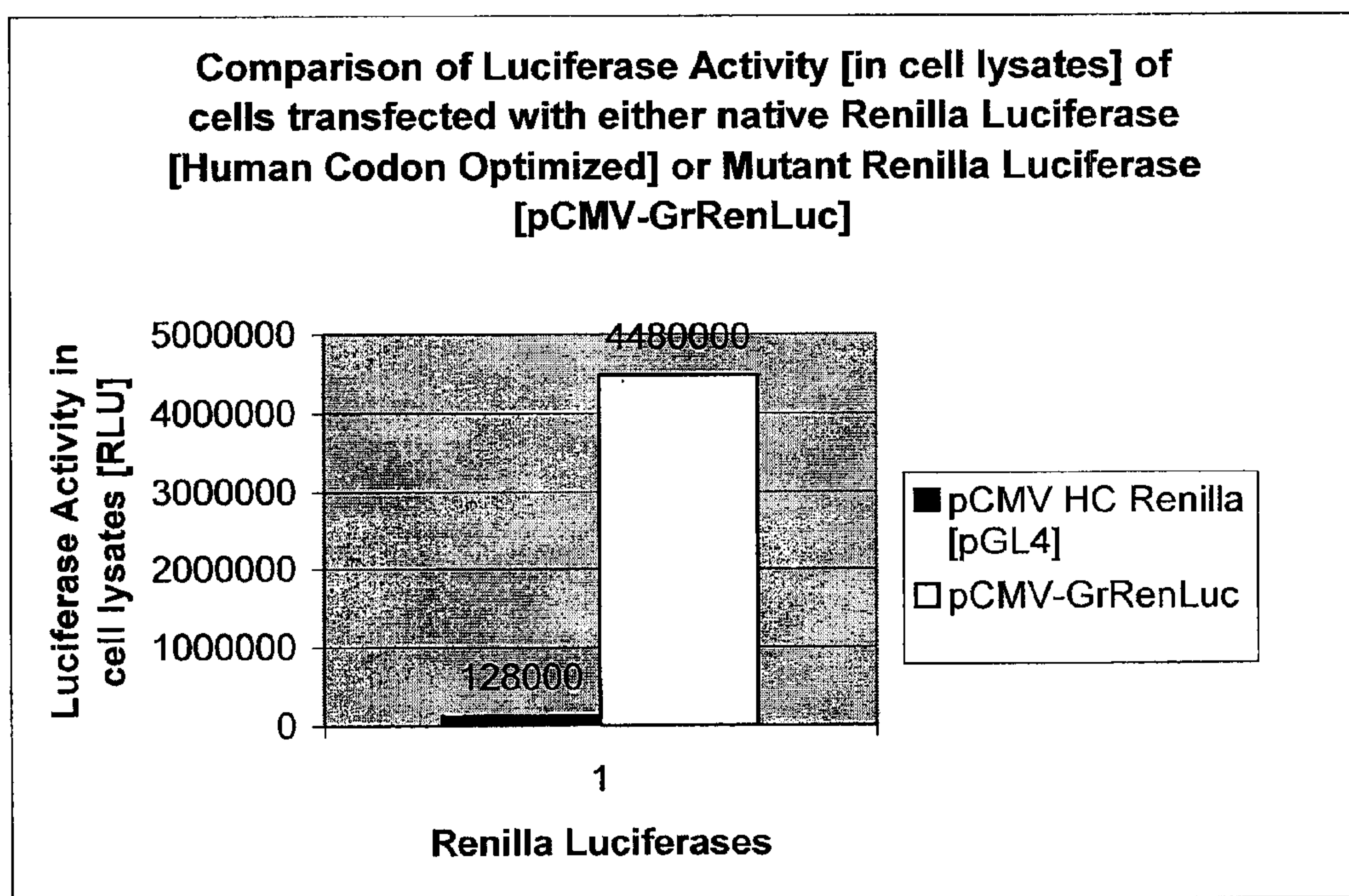




FIG. 8

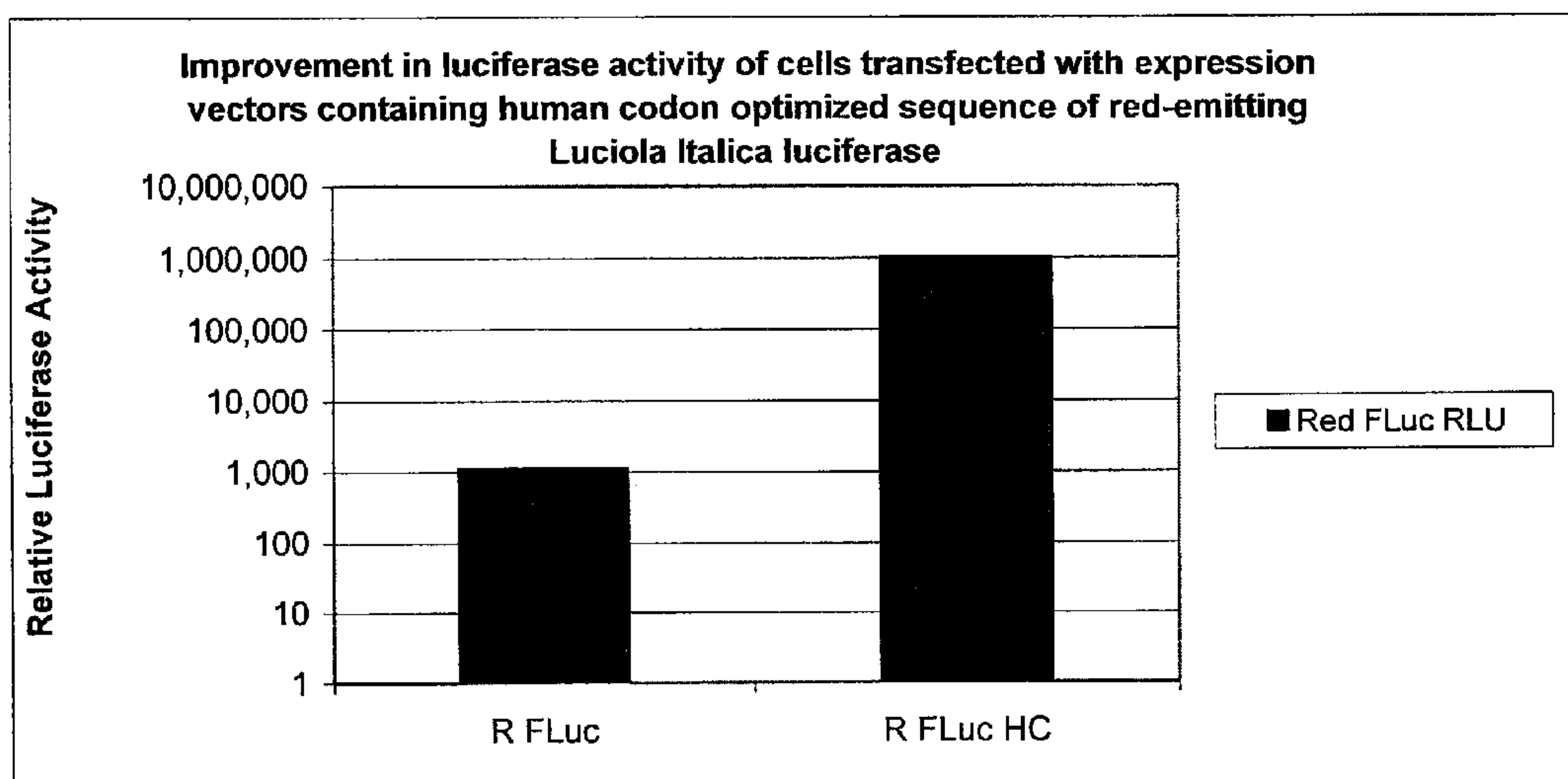


FIG. 9

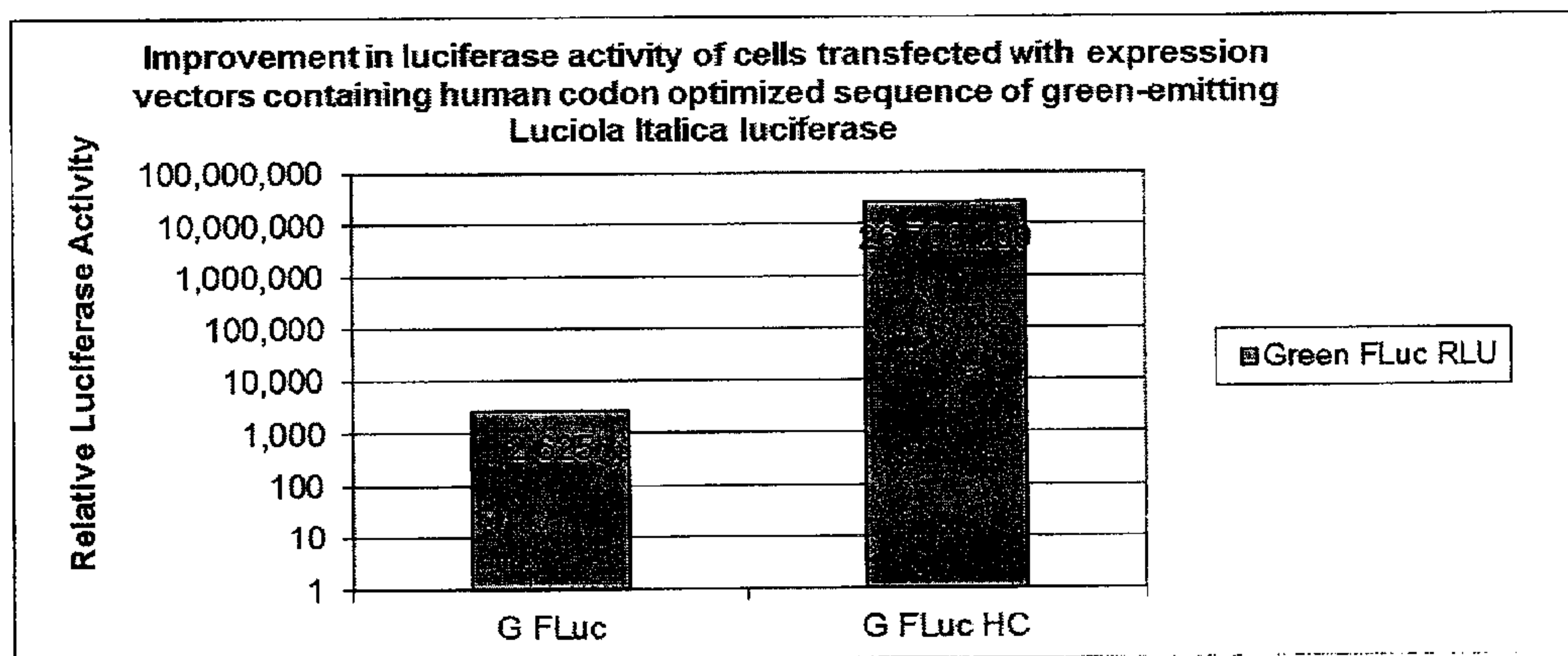




FIG. 10

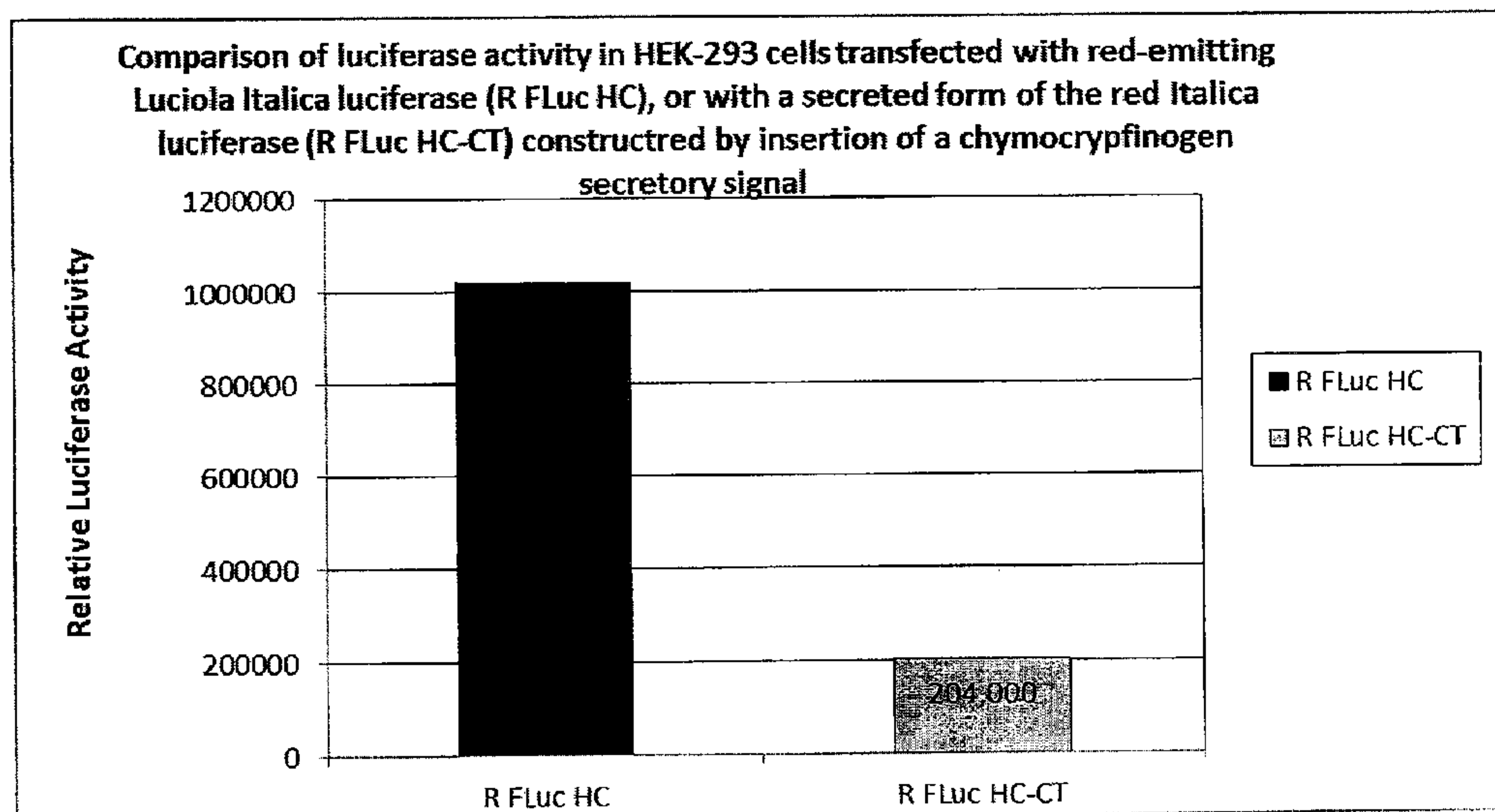


FIG. 11

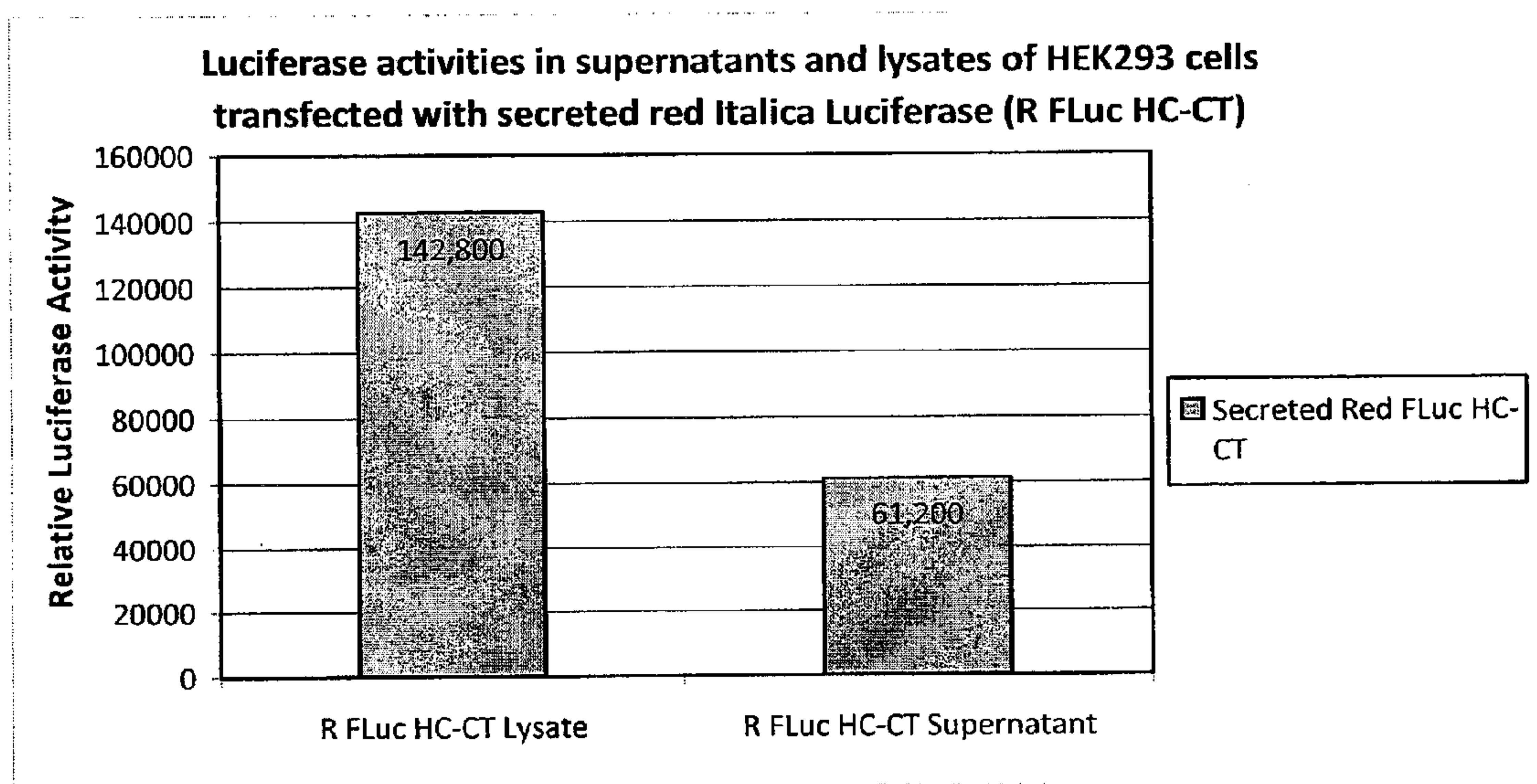




FIG. 12A

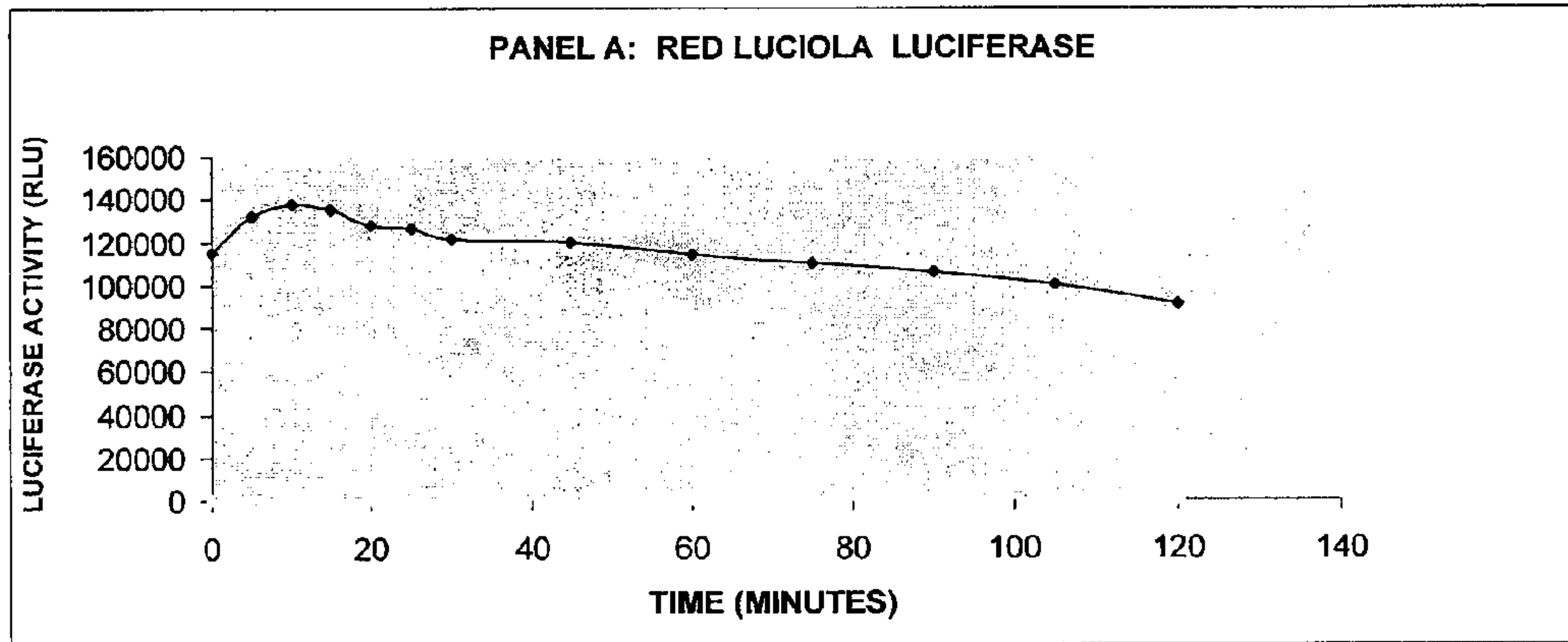


FIG. 12B

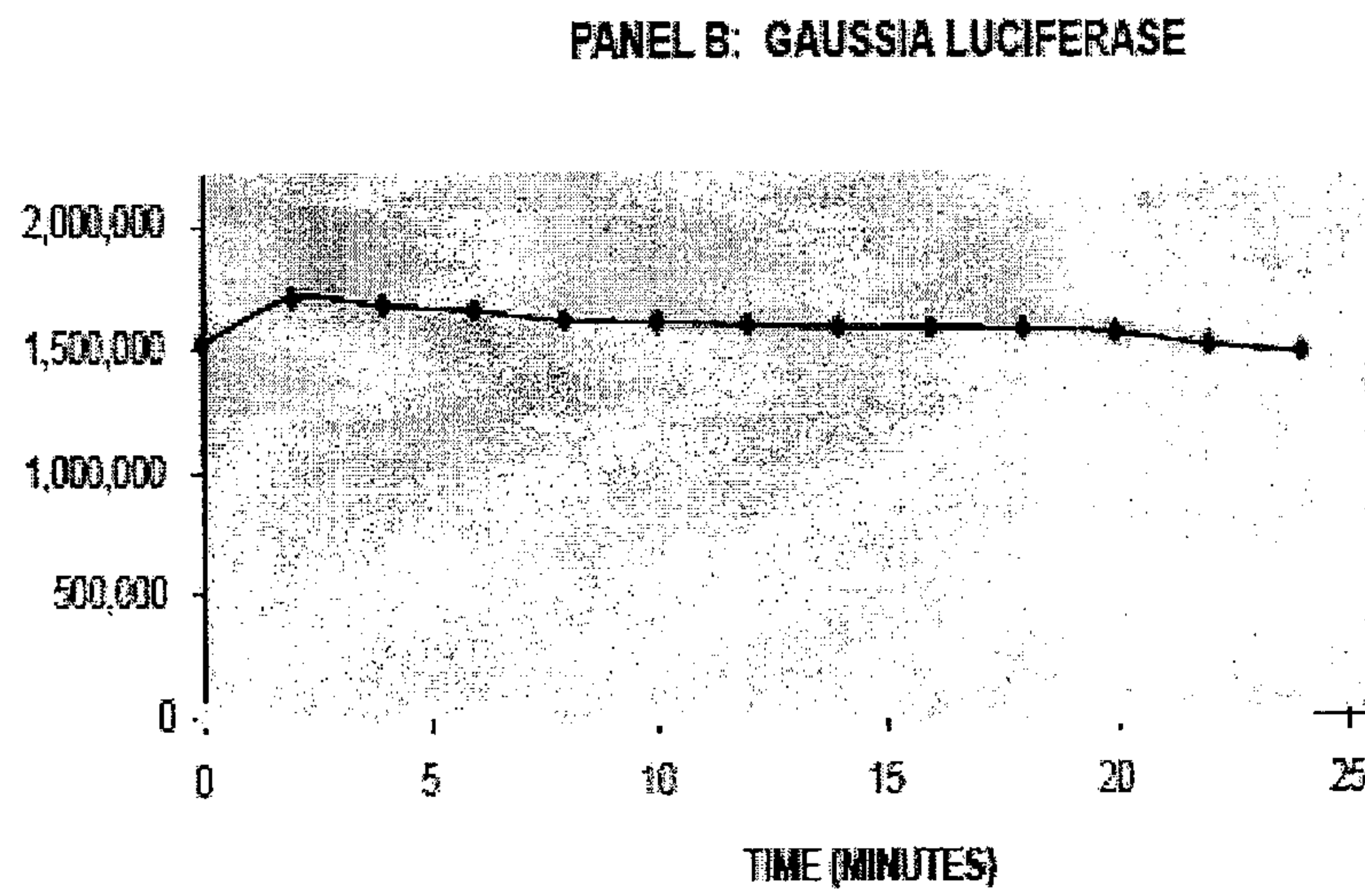


FIG. 12C

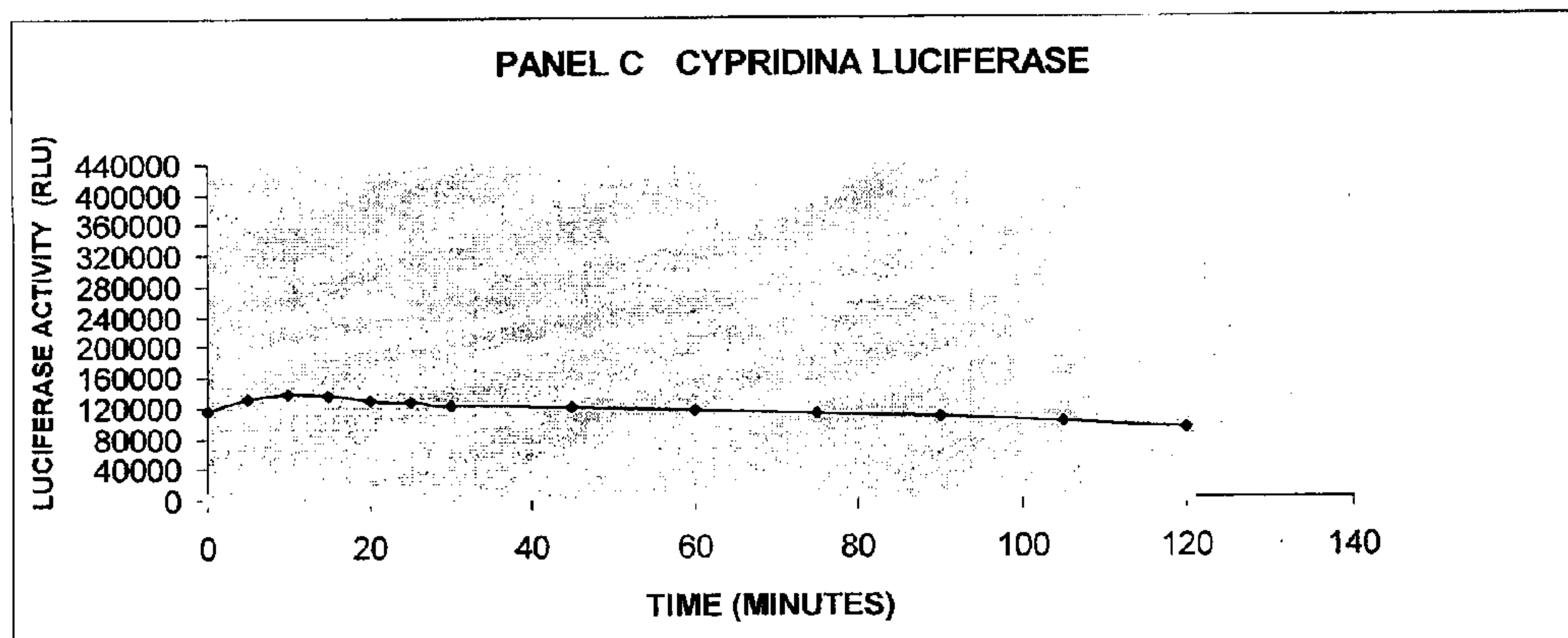


FIG. 12D

PANEL D: GREEN RENILLA LUCIFERASE

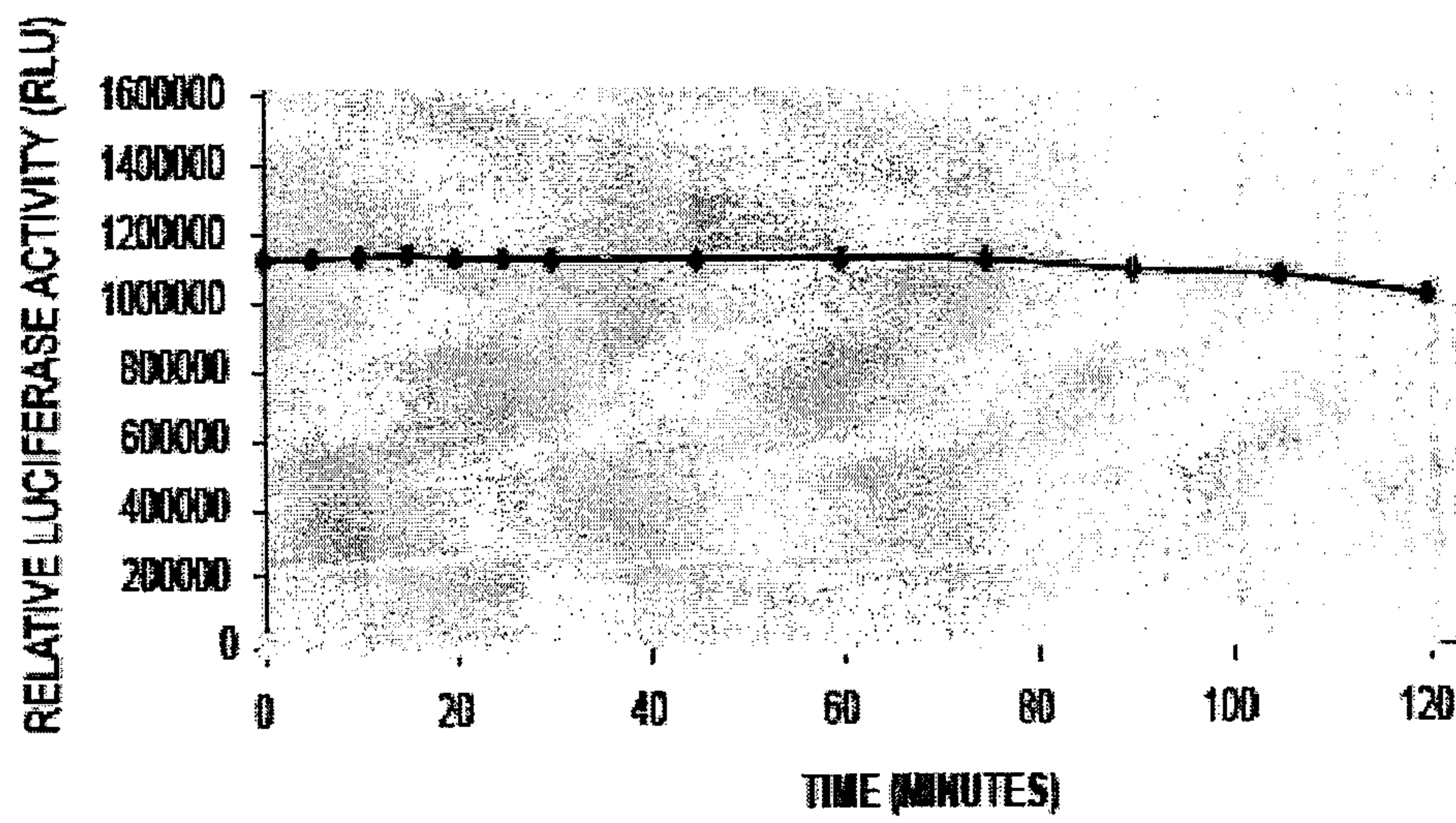




FIG. 13A

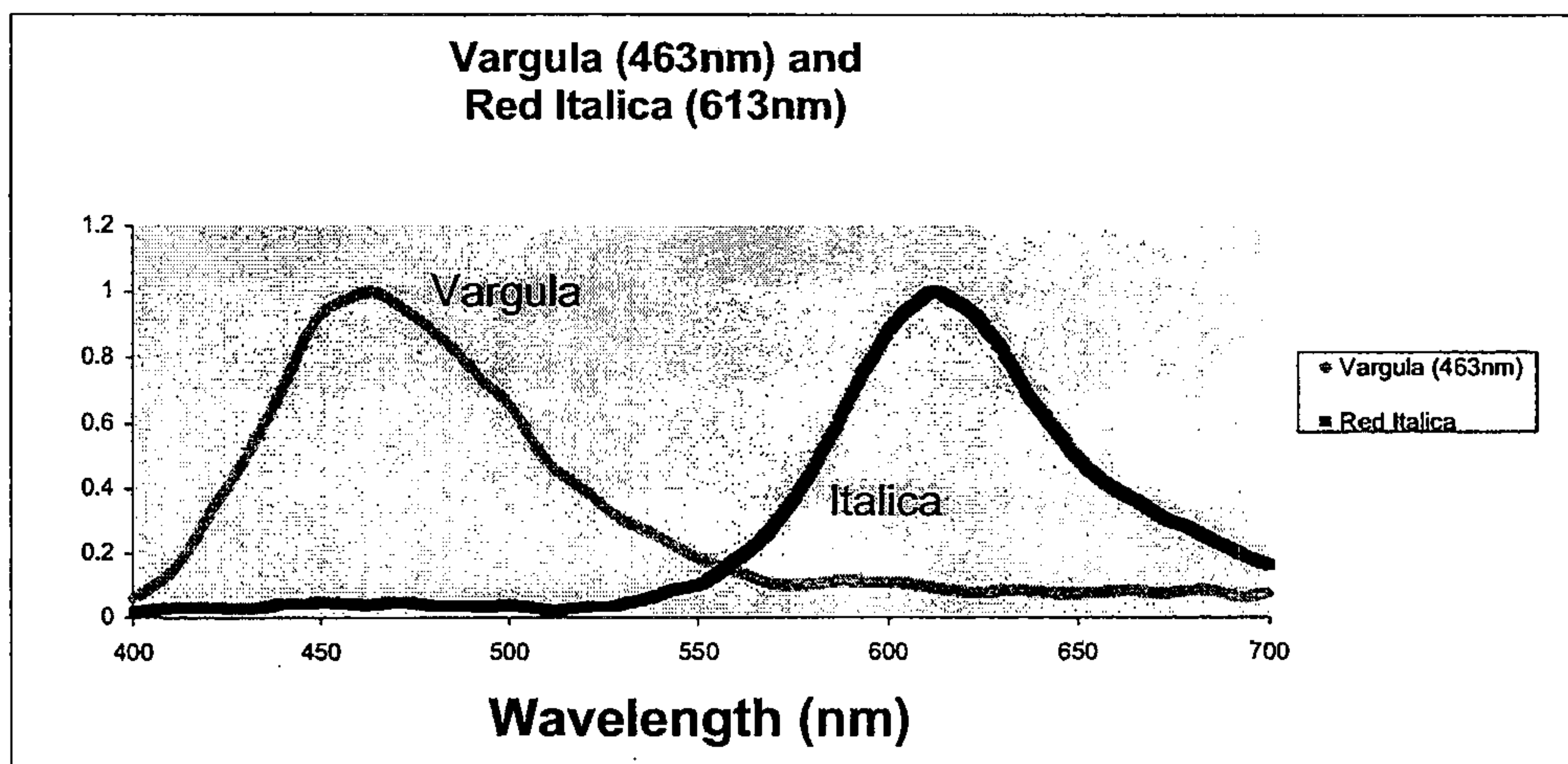


FIG. 13B

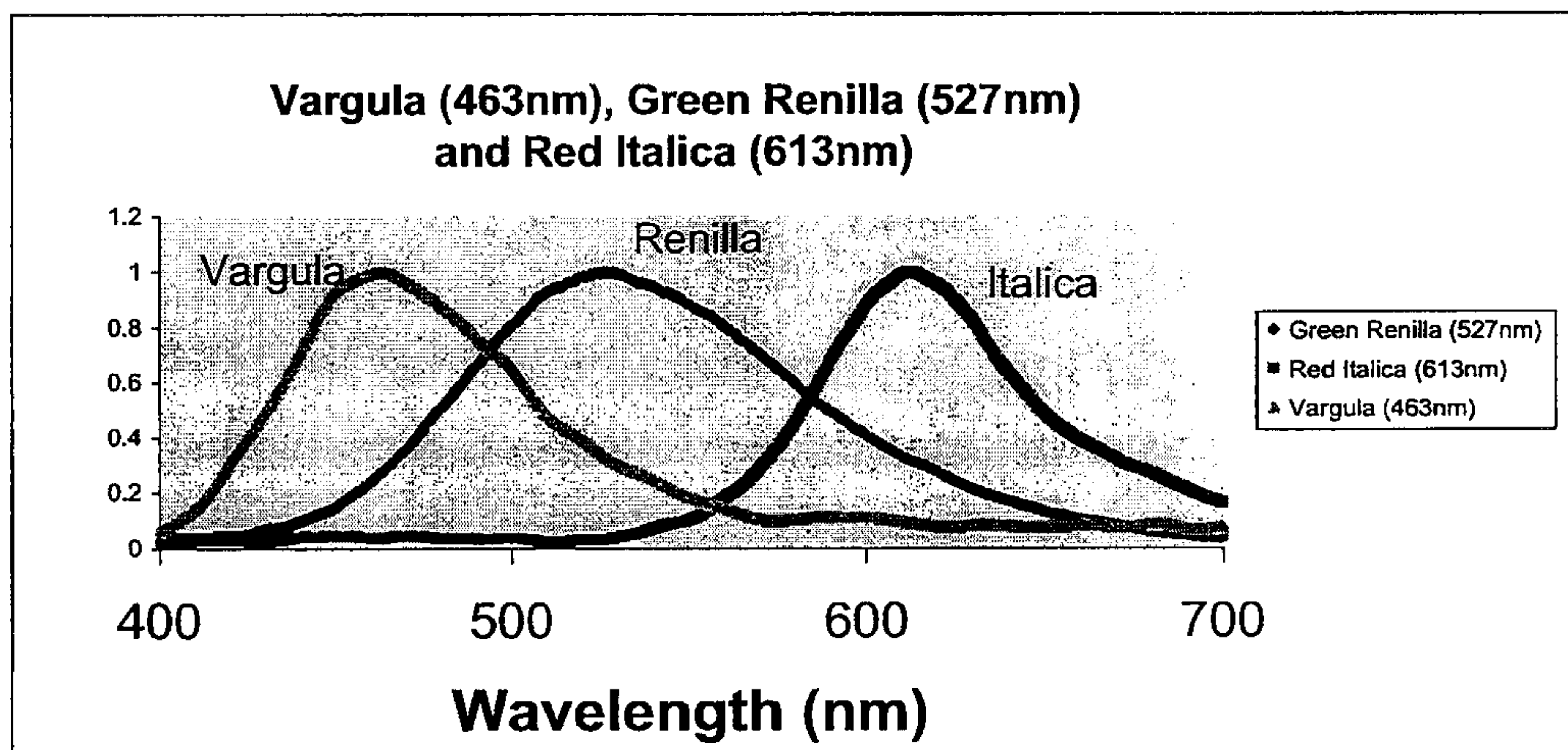


FIG. 14

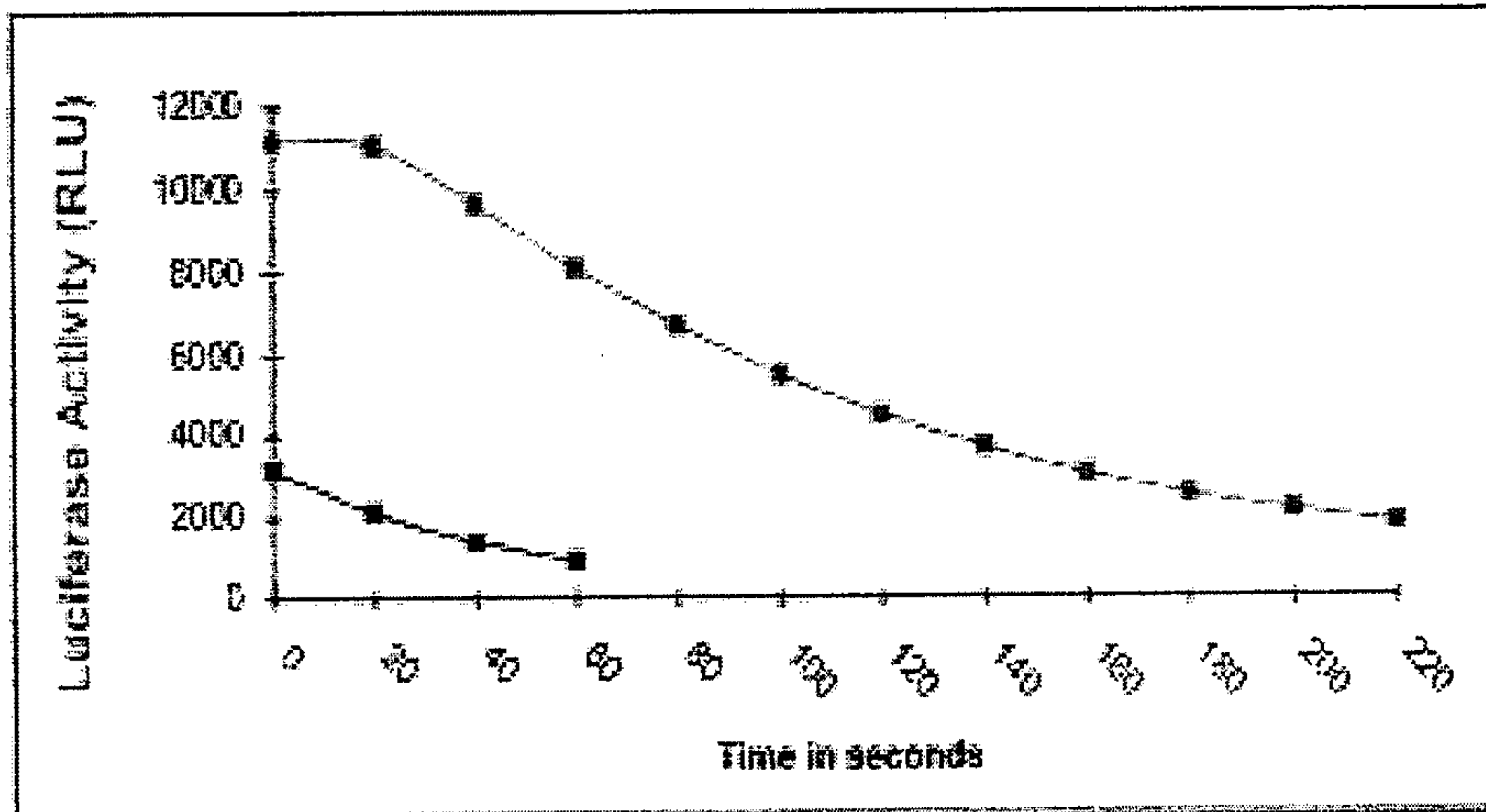




FIG. 15A

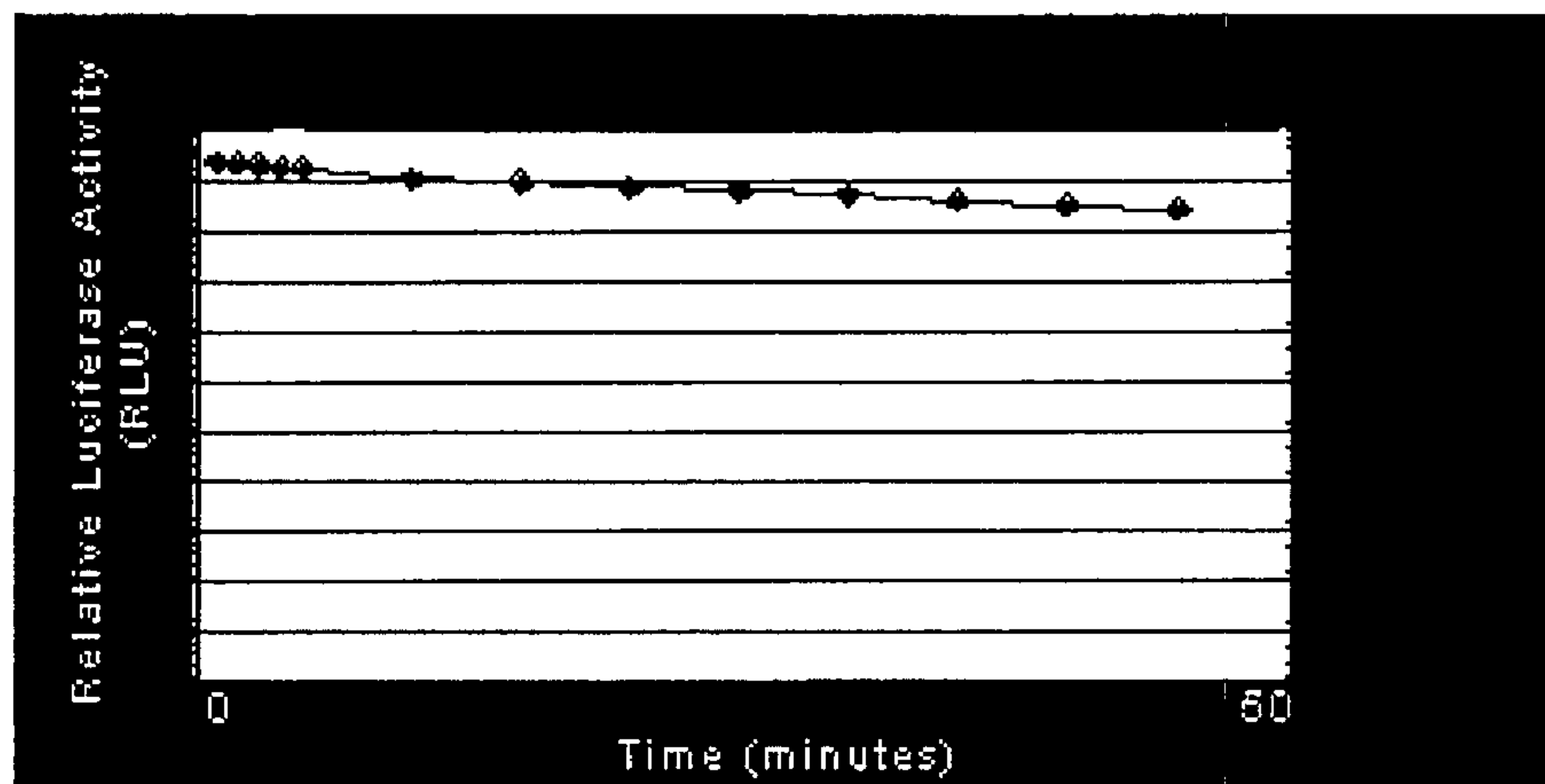


FIG. 15B

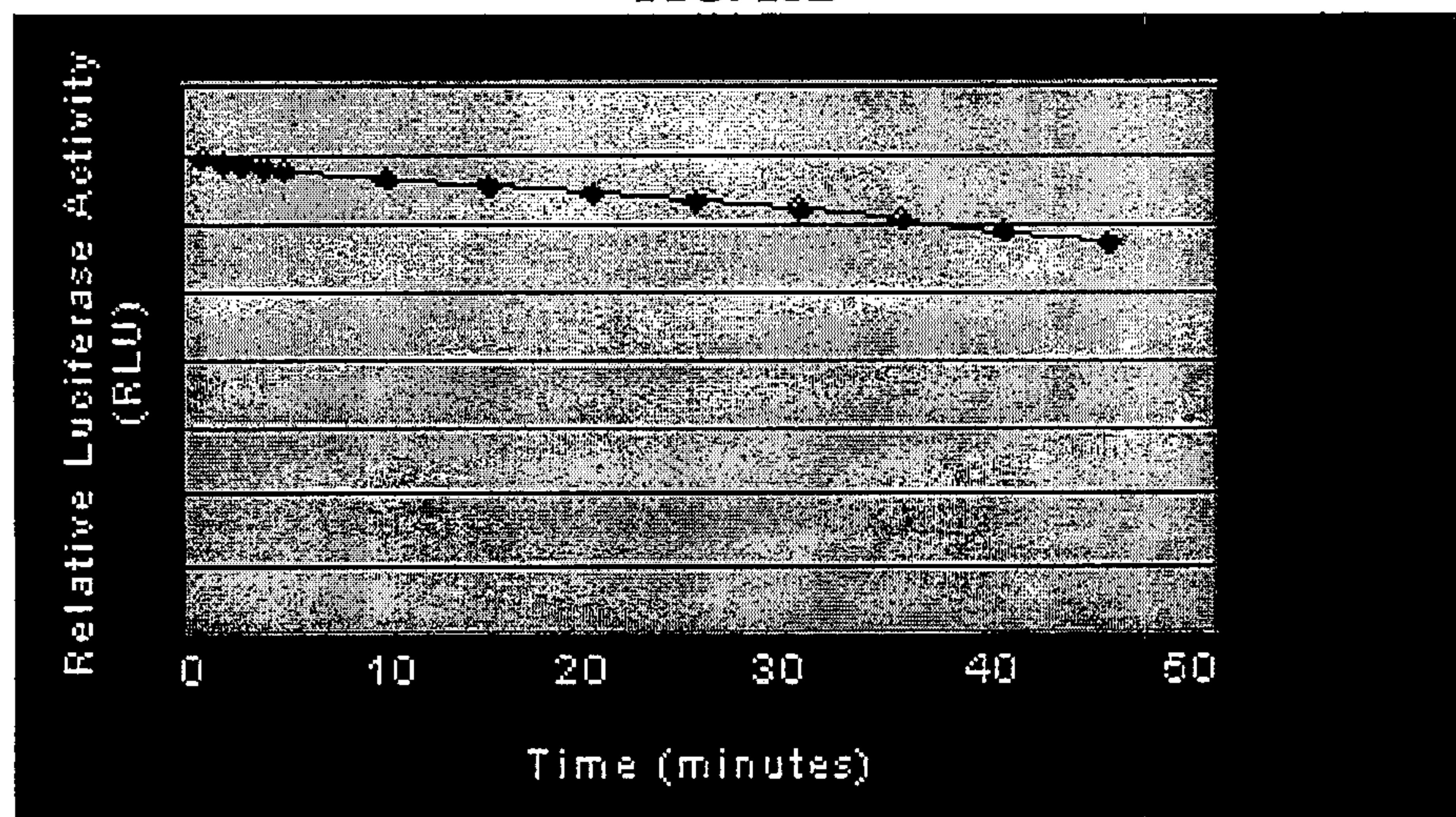


FIG. 16

Firefly Luciferase Assay reagent (FLAR-1)

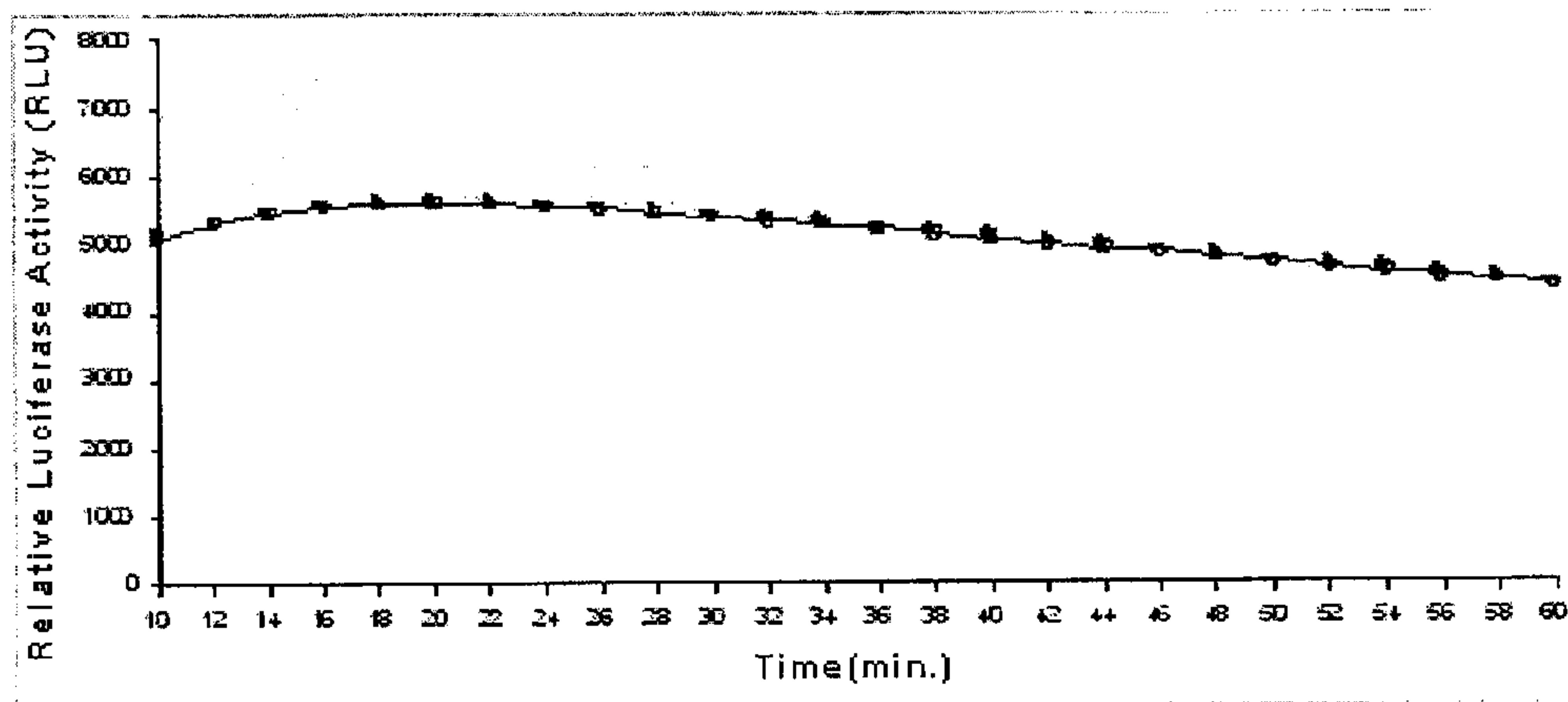


FIG. 17

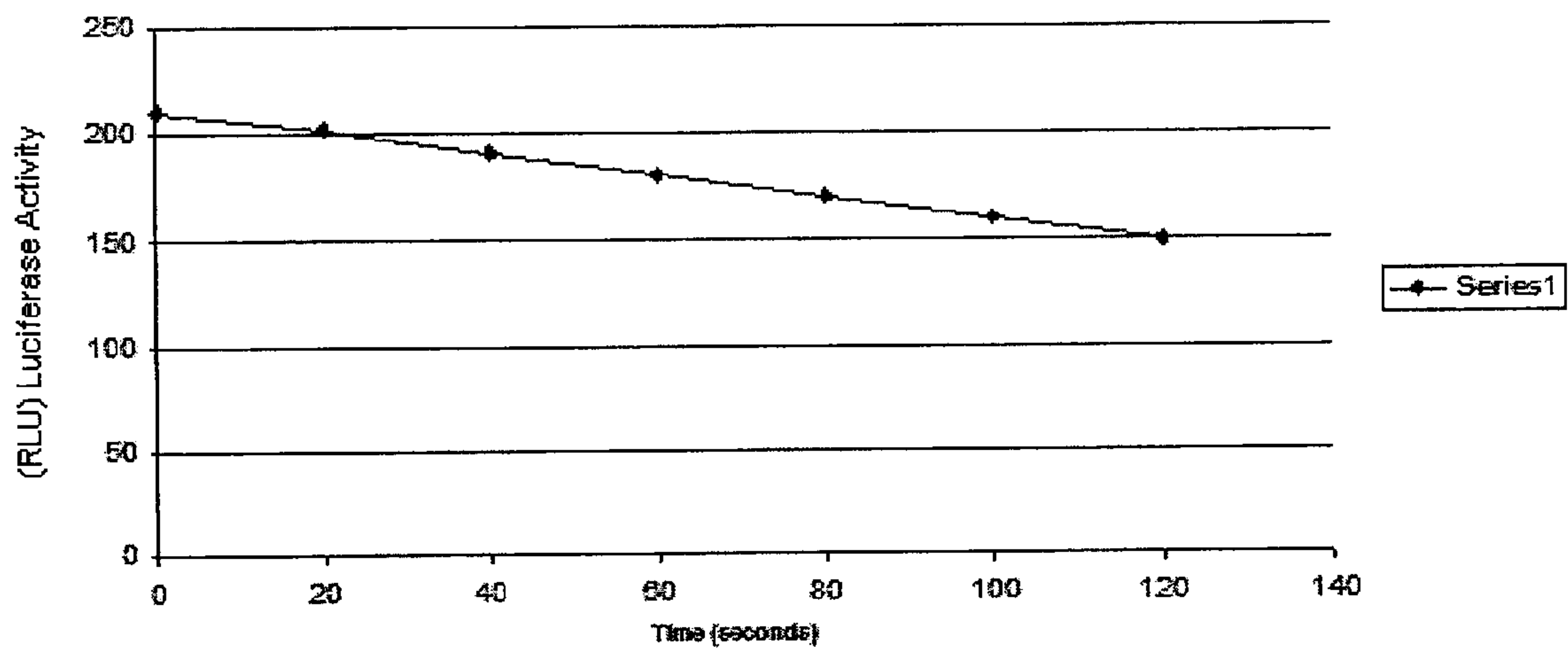




FIG. 18

Vargular Assay

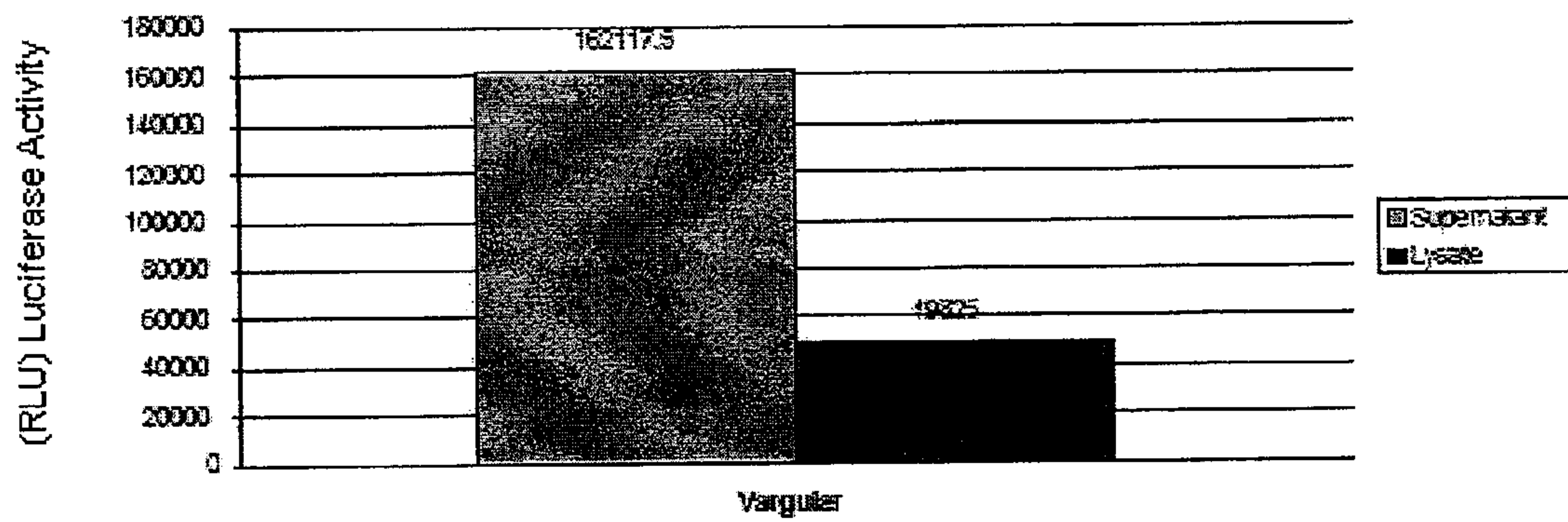


FIG. 19

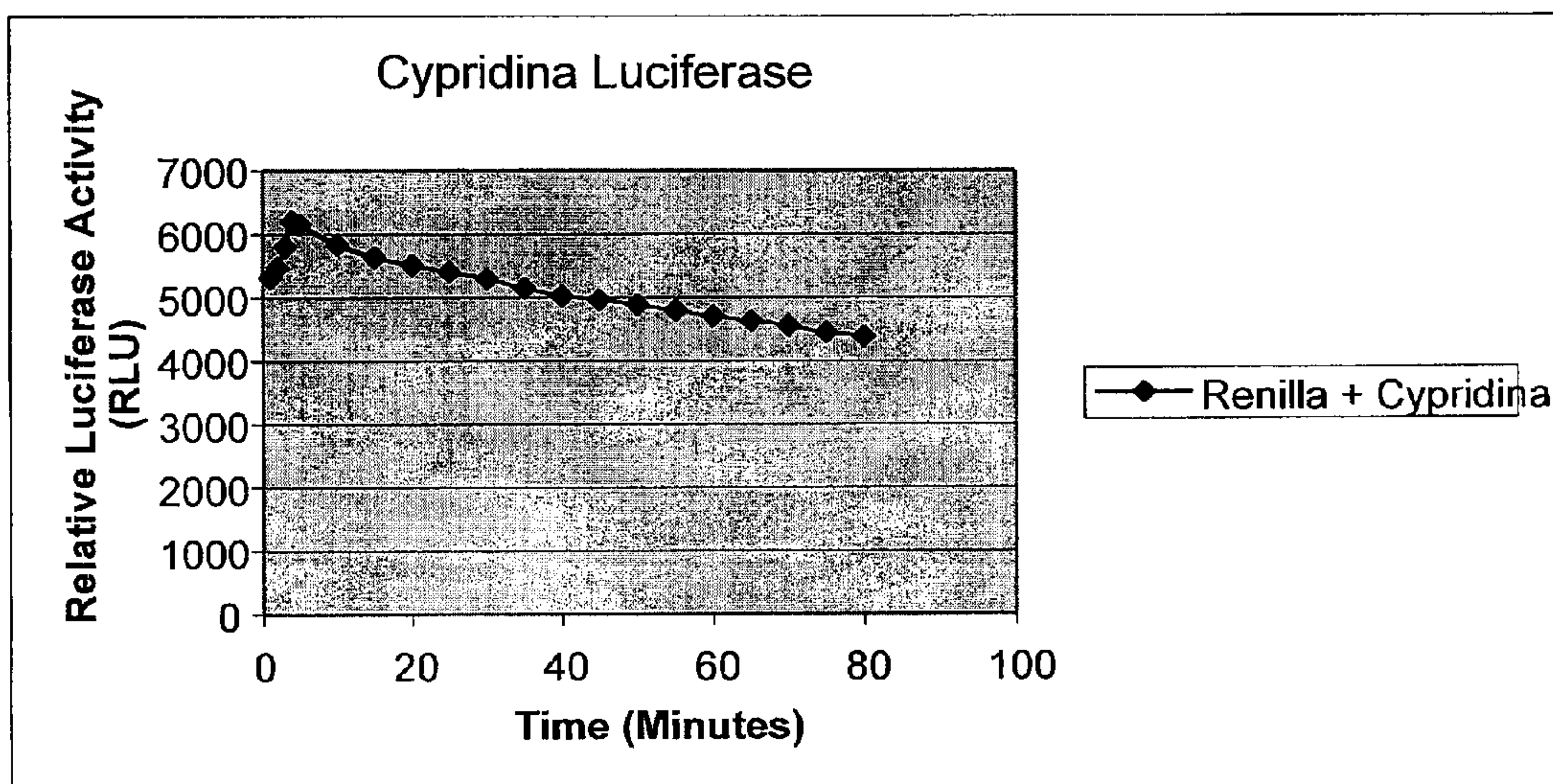




FIG. 20A

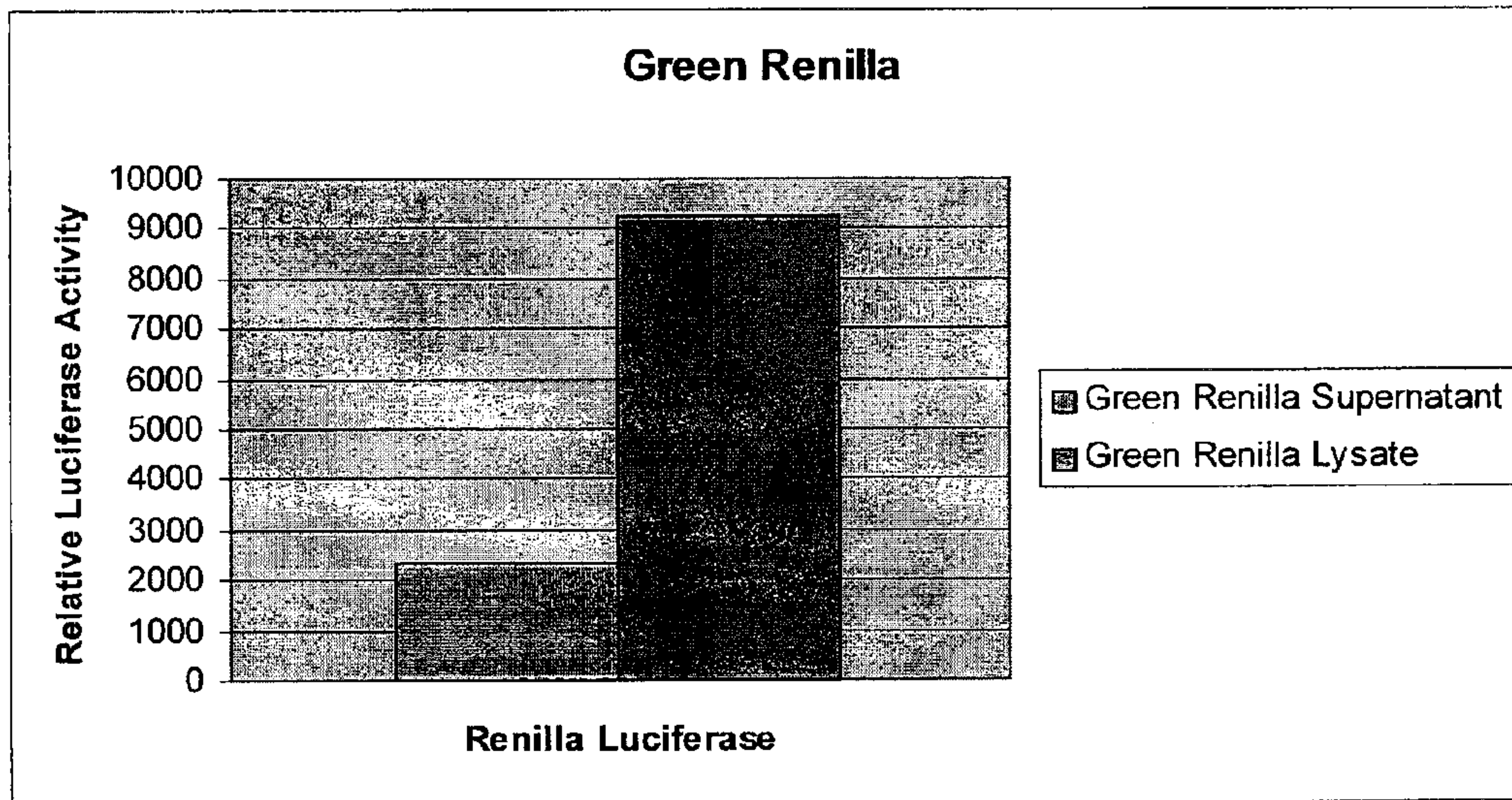


FIG. 20B

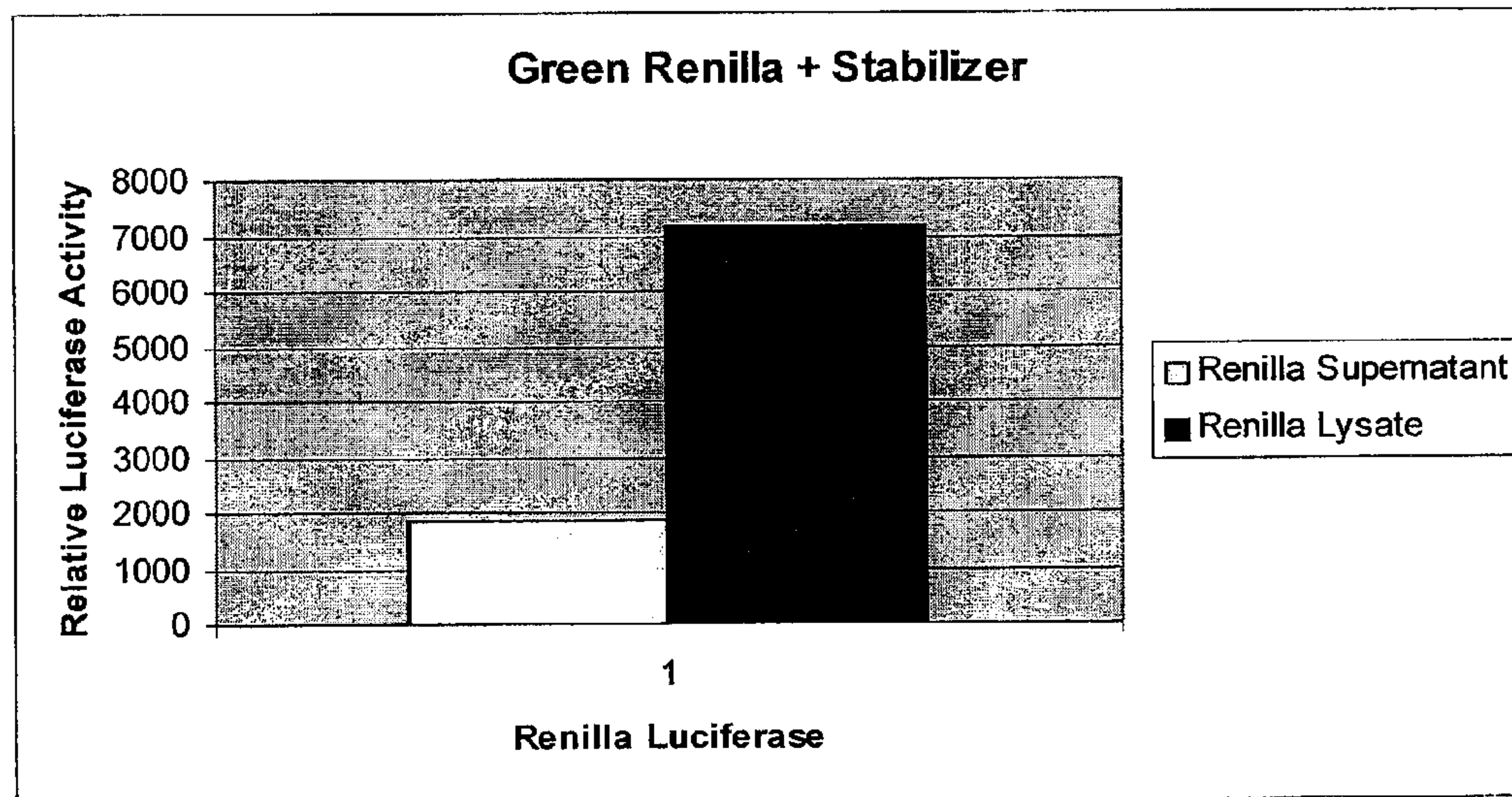




FIG. 21

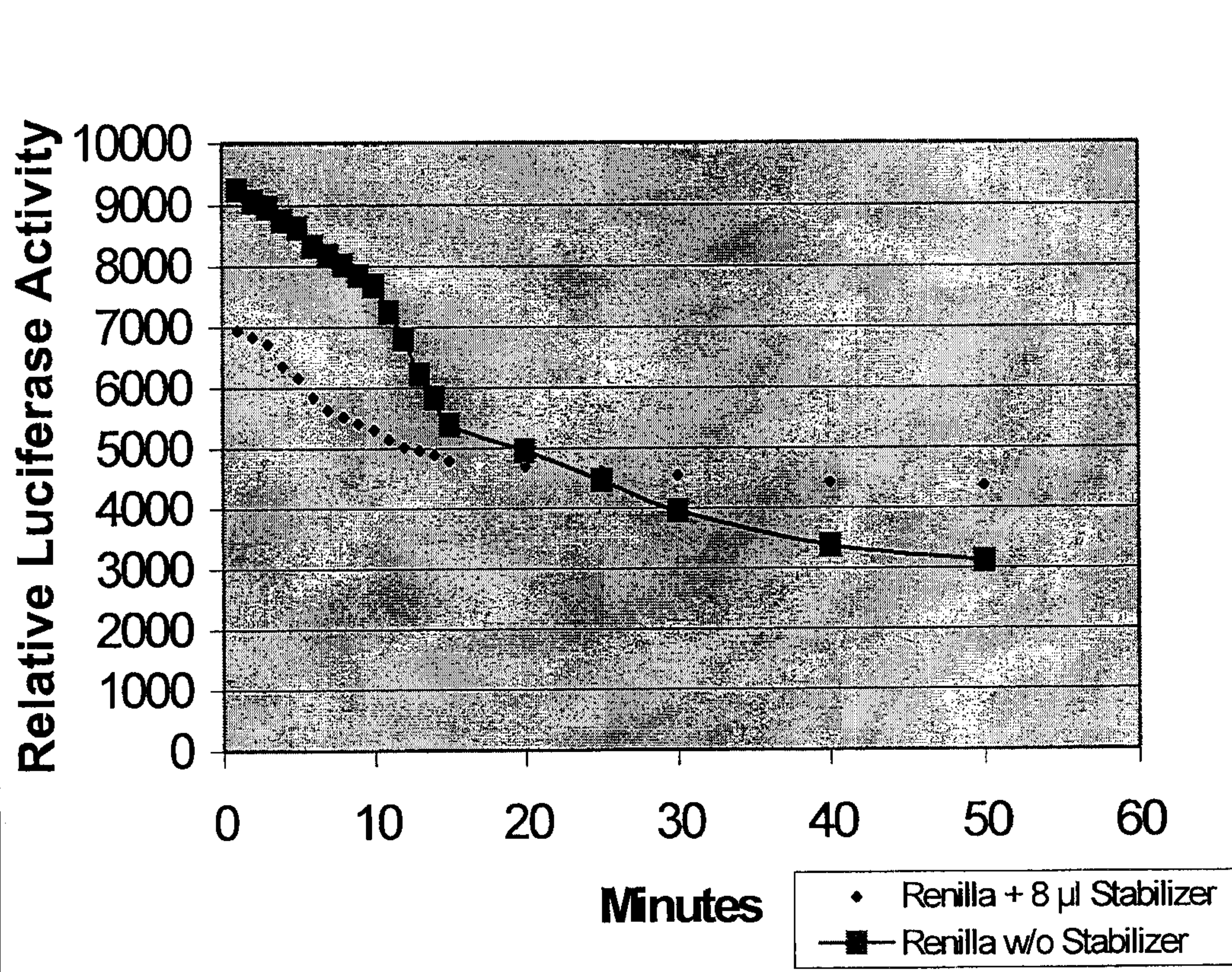




FIG. 22

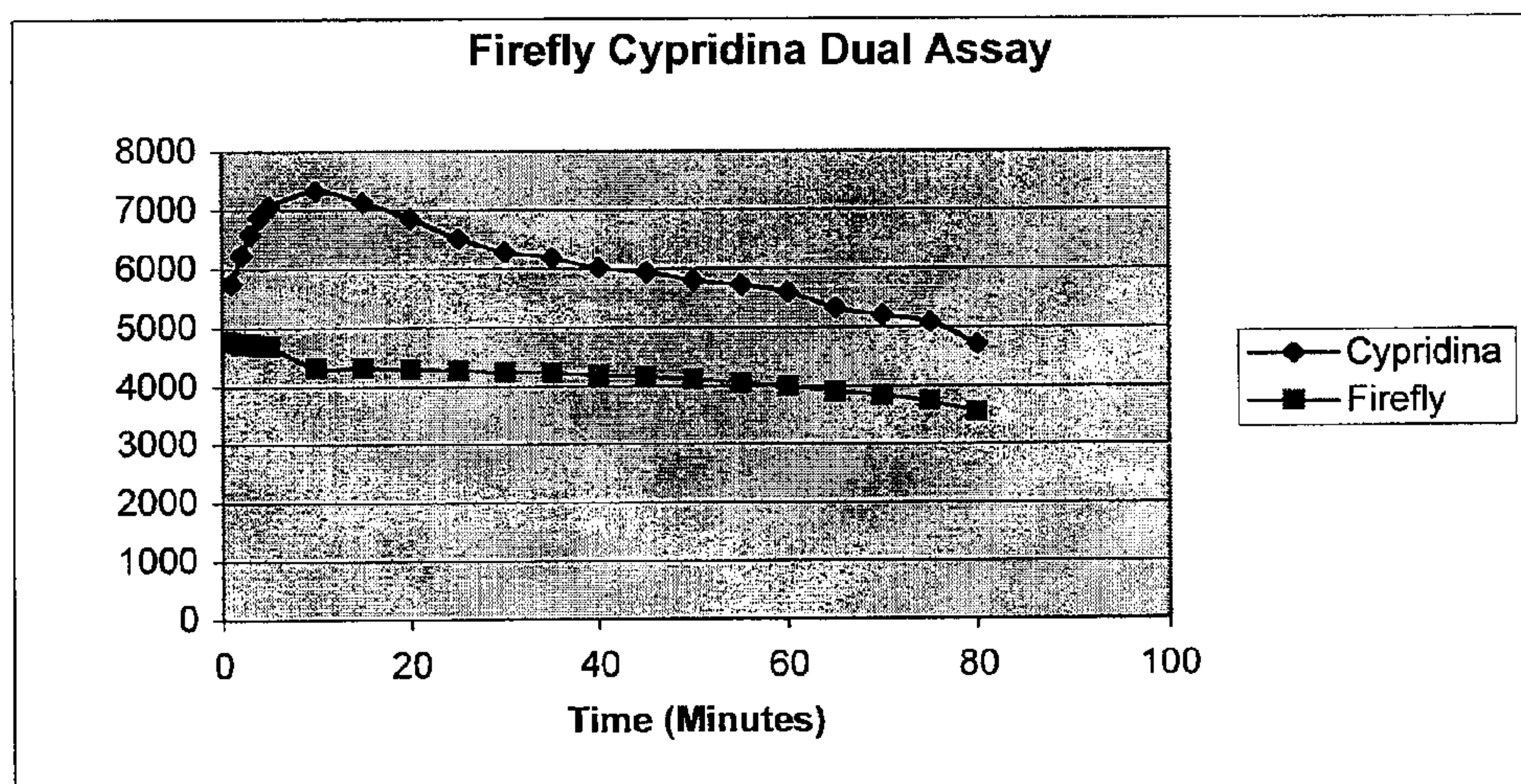


FIG. 23A

Dual Assay for Cypridina-Renilla Luciferase (DLAR-5)

Panel A

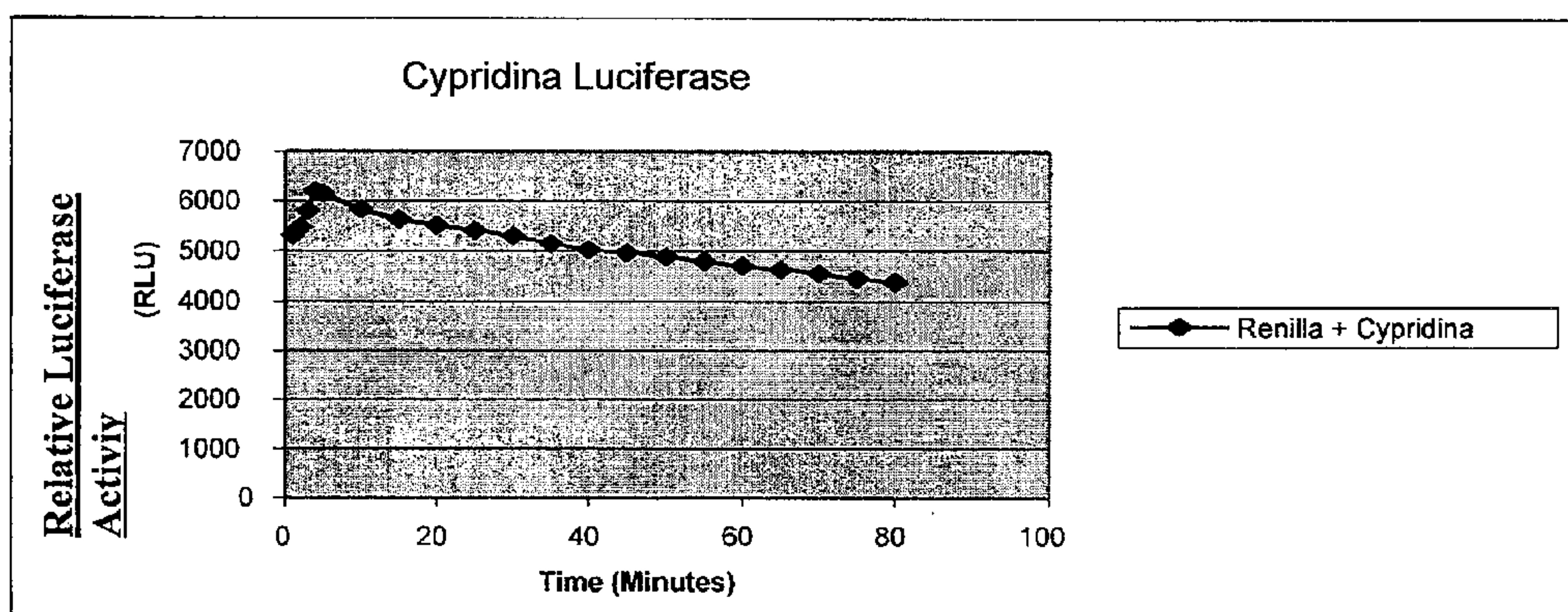


FIG. 23B

Panel B

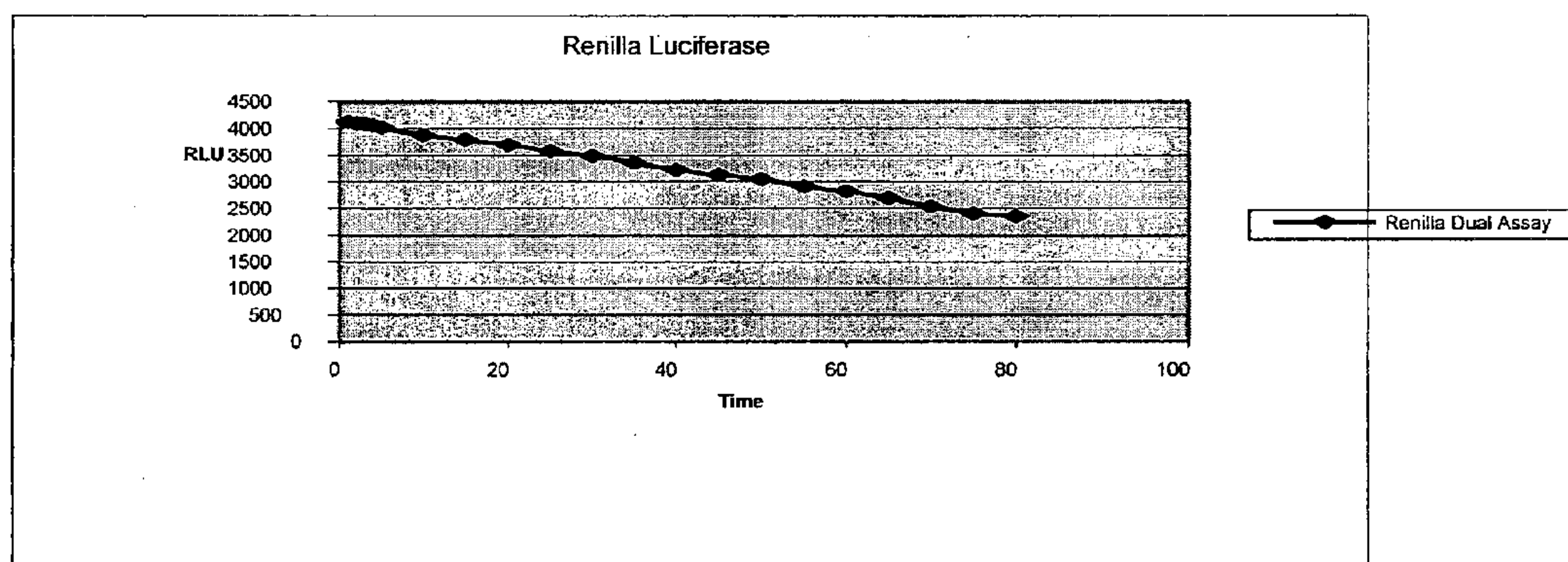




FIG. 24

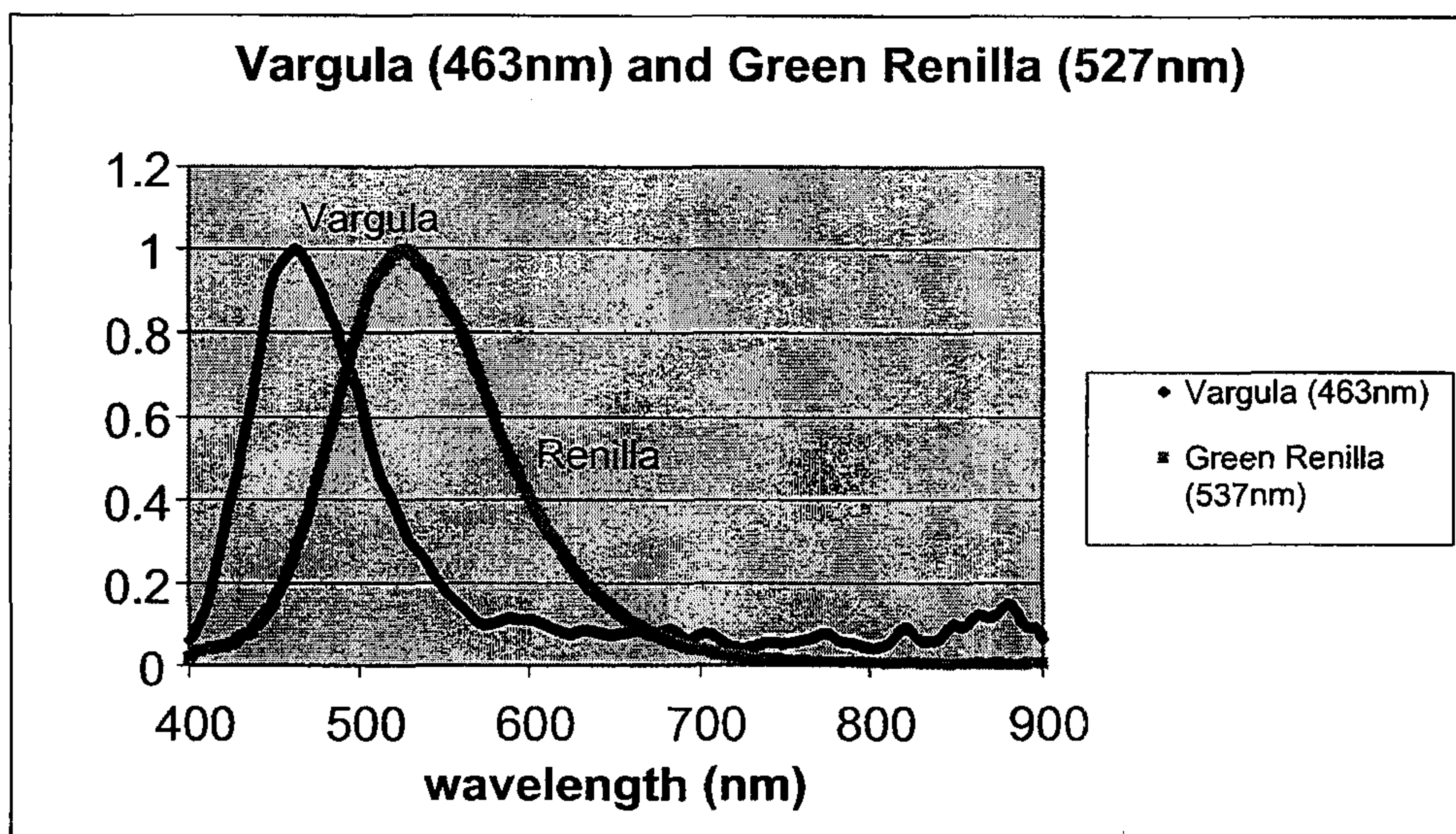


FIG. 25

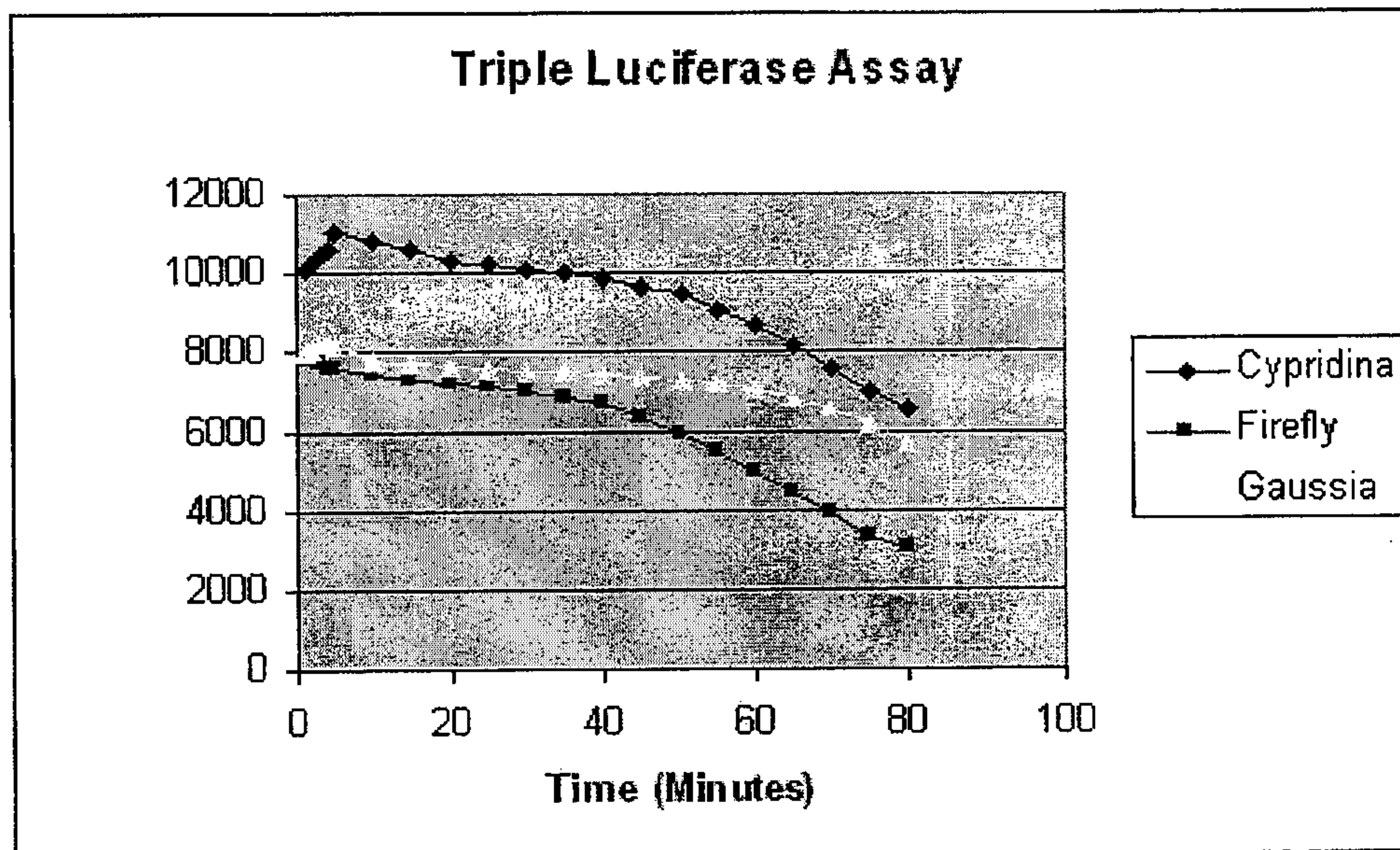


FIG. 26

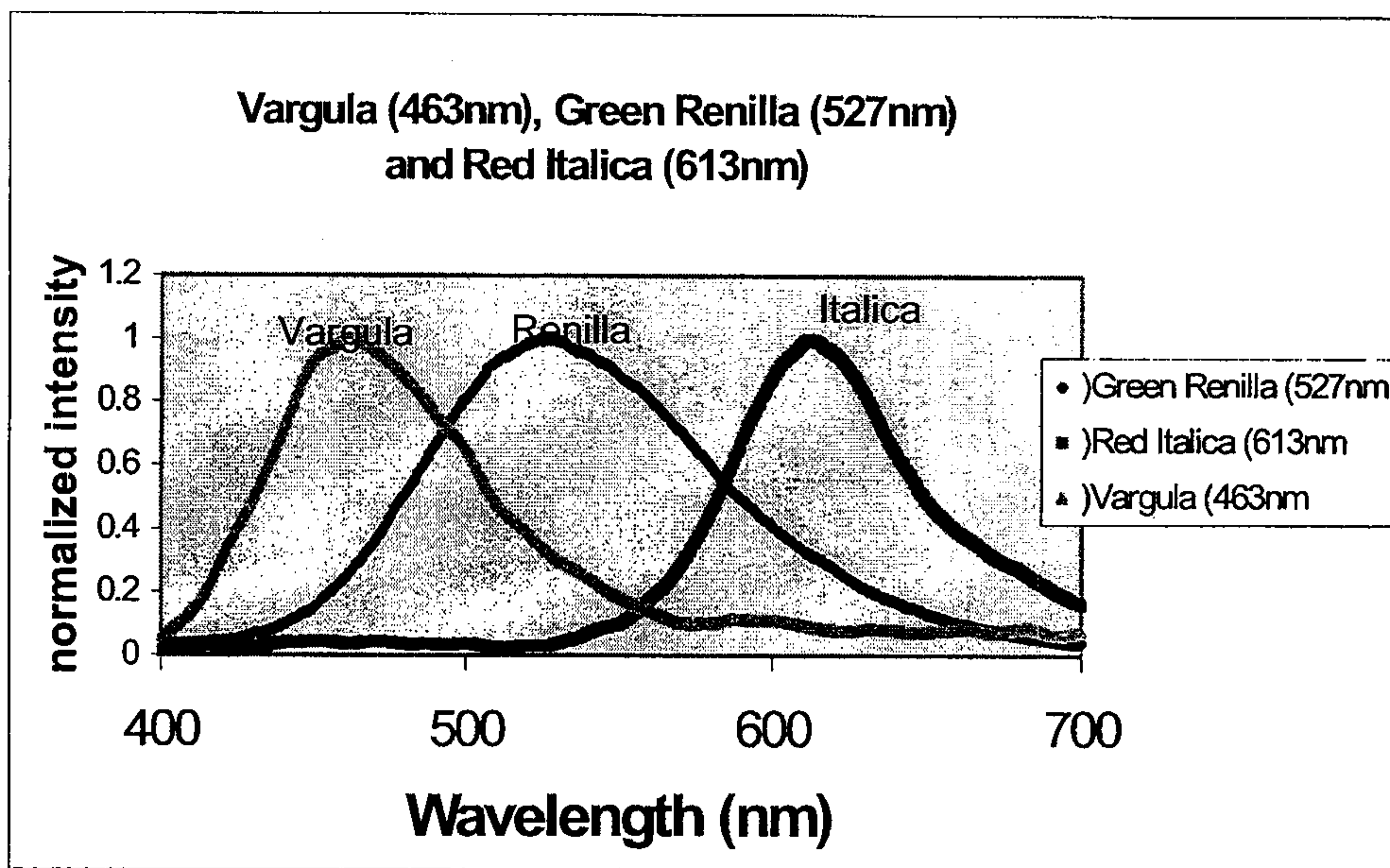




FIG. 27A

CMV Red Firefly Luciferase plasmid sequence:

## ORIGIN

1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg  
61 ccgcatagtt aagccagtat ctgctccctg ctgtgtgtt ggaggtcgct gagtagtgcg  
121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc  
181 ttagggtag gcgtttgcg ctgctcgcg atgtacgggc cagatatacg cgttgacatt  
241 gattattgac tagttattaa tagtaatcaa ttacgggggc attagttcat agcccatata  
301 tggagtccg cgttacataa ctacggtaa atggcccgcc tggctgaccg cccaacgacc  
361 cccgccatt gacgcaata atgacgtatg ttccatagt aacgccaata gggactttcc  
421 attgacgtca atgggtggac tattacggg aaactgcca ctggcagta catcaagtgt  
481 atcatatgcc aagtacgccc cctatgacg tcaatgacgg taaatggccc gcctggcatt  
541 atgccagta catgacctta tgggacttc ctactggca gtacatctac gtattagta  
601 tcgctattac catggtgatg cggtttggc agtacaatca tgggctgga tagcggttg  
661 acicacgggg attccaagt cccaccca ttgacgtcaa tgggagttg tttggcacc  
721 aaaatcaacg ggactttcca aatgtcgtg acaactccgc cccattgacg caaatgggcg  
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca  
841 ctgctactg gctatcgaa attaatcga ctactatag ggagaccca gcttggacc  
901 gagctcggat ccagccacca tggaaacaga aagagaagaa aacgttgtct acggcccact  
961 gccattctac ccgatcgagg agggctctgc cggcatcaa tgcacaagt acatgcaaca  
1021 atacgccaag ctggcgcca tcgctctcag taacgcccctg acaggcgtcg acatcagta  
1081 ccagcagtac ttgacatca cgtgcagact cgccgaggct atgaagaact acggcatgaa  
1141 gccagaagga cacatcgtc tctgtagcga gaactgcgaa gagttctca ttctgttct  
1201 ggctggtctt tacatcggag ttacagtcgc gccaactaac gaaattata cacttagaga  
1261 gctgaaccac agtctgggga tagccaacc tactatcgtt ttcttagca ggaagggcct  
1321 gccaaagtg ctgagggtc agaagaccgt gacttgcac aaaaccattg tcatcctgga  
1381 cagtaaggtc aactcggcg gttatgactg cgtagagacc ttcattaaga aacacgtcga  
1441 gctgggctt cctgccacct catttggcc catcgacgtc aaagaccgga agcaccacat  
1501 tgctctgctt atgaactctt ccggttccac agggctgccc aaaggagtag agatcactca  
1561 cgaggccctg gtcacgagat tctctcagc taaggaccct atatacggca atcaggtggc  
1621 cccaggtacc gctatcctga ctgtcgtgcc ttccaccac ggcttcggaa tgttactac  
1681 tttgggtac ttgctcgcg gttaccgat tgcattgct actaagttcg acgaggagct  
1741 ttctcgcgc acactcagg attacaagt cactacagta atctgggtc cgacactgtt  
1801 cgcaattctt aataggctc agctcctga taagttgac ctcttaacc tgactgaaat  
1861 agccagcgtt ggtgctccac ttgccaagga gatcggcgag gctgtgcaa gaagattcaa  
1921 cctcccaggc gtccggcagg gatatggact caccgagact accagtgcct ttatcatcac  
1981 tctaagggc gacgacaagc cgggagccag cggcaaggtc gtgcctctgt tcaaggtgaa  
2041 gattattgac ctcgatacca agaaaacgtt ggggtgcaac agacggggag aaatctcgt  
2101 gaaaggacca tctctatgt tgggatacac gaacaatcct gaagccacca gagaaactat  
2161 tgacgaggaa ggctggctgc acacgggtga catcgggtac tacgacgagg atgagcactt  
2221 cttatagtc gaccgcctga aatctctcat taagtataaa ggataccaag tgccaccagc  
2281 tgaactggag tctgtctcc tgcaacacc taacattaga gatgctggg tggccgggg  
2341 tccgacagc gaggcaggcg agctgcctgg agccgtcgtt gtgatggaaa agggaaagac  
2401 aatgactgag aaagaaatc tagactatgt aaactcccag gtggtaacc acaagcggct  
2461 gaggggccc gtgcggctc tagatgaagt cccaagggg ctacacaggaa agatcgacgc  
2521 gaaagtatc agggagatac tcaagaaacc tcaagcagg gggtagtcta gaaataattc  
2581 ttactgtcat gccaagtaag atgctttct gtgtgcaat agcaggcatg ctggggatgc  
2641 ggtgggctct atggctctg aggcgaaag aaccagctgg ggctctaggg ggtatccca  
2701 cgcgccctgt agcggcgc at taagcgcggc ggggtggtg gttacgcgca gcgtgaccgc  
2761 tacactgcc agcgcctag cgcgcctcc ttgccttc ttccctct tctcgcac  
2821 gttcgcggc ttccccgc aagctctaaa tggggcatc ccttaggg tccgattag  
2881 tgcttacgg cacctcgacc ccaaaaaact tgattaggg gatggtcac gtatggggc  
2941 atgcctga tagacgggtt ttgccctt gacgtggag tccacgtct ttaatagtg  
3001 actctgtc caaactggaa caaactcaa ccctatctc gtctattct ttgattata  
3061 agggatttg gggattcgg cctatgggt aaaaaatgag ctgattaac aaaaattaa



FIG. 27B

3121 cgcgaaattaa ttctgtggaa tgtgtgtcag ttaggggtgtg gaaagtcccc aggctcccca  
3181 ggcaggcaga agtatgcaaa gcatgcatct caattagtca gcaaccagggt gtggaaagtc  
3241 cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt cagcaacccat  
3301 agtccccgcc ctaactccgc ccatccccgc cctaactccg cccagttccg cccattctcc  
3361 gccccatggc tgactaattt ttttattta tgcagaggcc gaggccgcct ctgcctctga  
3421 gctattccag aagtagtgag gaggctttt tggaggccta ggcttttga aaaagctccc  
3481 gggagctgt ataccattt tcggatctga tcaagagaca ggatgaggat cgtttcgcat  
3541 gattgaacaa gatggattgc acgcaggctc tccggccgct tgggtggaga ggctattcgg  
3601 ctatgactgg gcacaacaga caatcggctg ctctgatgcc gccgtgttcc ggctgtcagc  
3661 gcagggggcg ccggtcttt ttgtcaagac cgacctgtcc ggtgccctga atgaactgca  
3721 ggacgaggca gcgcggctat cgtggctggc cacgacgggc gttccttgcg cagctgtgct  
3781 cgacgtgtc actgaagcgg gaagggactg gctgtattg ggcaagtgc cggggcagga  
3841 tctctgtca tctcacctg ctctgccga gaaagtatcc atcatggctg atgcaatgcg  
3901 gcggctgcat acgctgtatc cggctacctg cccattcgac caccaagcga aacatcgcat  
3961 cgagcgagca cgtactcggg tggagccgg tcttctgat caggatgatc tggacgaaga  
4021 gcatcagggg ctgcgccag ccgaactgtt cgccaggctc aaggcgcgca tgcccagcgg  
4081 cgaggatctc gtcgtgacct atggcgatgc ctgcttgcg aatatcatgg tggaaaatgg  
4141 ccgctttct ggattcatc actgtggccg gctgggtgtg gcggaccgct atcaggacat  
4201 agcgttggct acccgtgata ttgctgaaga gcttggcggc gaatgggctg accgcttct  
4261 cgtgctttac ggtatcgccg ctcccgattc gcagcgcac gccttctatc gccttctga  
4321 cgagttctc tgagcgggac tctggggtc gaaatgaccg accaagcgcg gcccaacctg  
4381 ccatcacgag atttcgattc caccgccgc tctatgaaa ggttgggctt cggaatcgtt  
4441 ttccgggacg ccgctggat gatcctccag cgcggggatc tcatgctgga gttctcgc  
4501 caccccaact tgttattgc agcttataat gttacaaat aaagcaatag catcacaat  
4561 ttcacaaata aagcatttt tcaactgcat tctagtgtg gttgtcca actcatcaat  
4621 gtatctatc atgtctgat accgtcgacc tctagctaga gcttggcgta atcatggtca  
4681 tagctgttc ctgttgaaa ttgtatccg ctcaaatc cacacaacat acgagccgga  
4741 agcataaagt gtaaagcctg ggggtcctaa tgagttagct aactcacatt aattgcgtg  
4801 cgctcactgc ccgcttcca gtcgggaaac ctgtcgtgcc agctgcatta atgaatcggc  
4861 caacgcgcgg ggagaggcgg ttgcgtatt gggcgtctt ccgcttctc gctcactgac  
4921 tcgctgcgt ccgtcgttcg gctgcggcga gcggtatcag ctactcaaa ggccgtaata  
4981 cggttatcca cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa  
5041 aaggccagga accgtaaaaa ggccgcgtg ctggcgttt tccataggct ccgccccct  
5101 gacgagcatc acaaaaatcg acgctcaagt cagagggtgc gaaaccgcg aggactataa  
5161 agataccagg cgttcccc tggaagctcc ctctgcgct ctctgttcc gaccctgccg  
5221 cttaccgat acctgtccg ctttctctt tcgggaagcg tggcgttct tcaatgctca  
5281 cgctgtagg atctcagttc ggttaggtc gttcgtcca agctgggctg tgtgcacgaa  
5341 cccccgtc agcccgaccg ctgcgcctta tccgtaact atcgtcttga gtccaaccg  
5401 gtaagacacg acttatgcc actggcagca gccactggt acaggattag cagagcagg  
5461 tatgtaggcg gtgtacaga gttctgaag tggggccta actacggcta cactagaagg  
5521 acagtattg gtatctgcg tctgtgaag ccagttacct tcggaaaaag agttggtagc  
5581 tctgatccg gcaaaaaac caccgtggt agcgggtgtt tttgtttg caagcagcag  
5641 attacgcgca gaaaaaagg atcaagaa gatccttga tctttctac ggggtctgac  
5701 gctcagtga acgaaaactc acgttaaggg atttgttca tgagattatc aaaaaggatc  
5761 ttcacctaga tcttttaa taaaaatga agttttaa caatctaaag tataatgag  
5821 taaactggt ctgacagta ccaatgctta atcagtgagg cacctatctc agcgatctg  
5881 ctattctgt catccatagt tgcctgactc cccgtcgtg agataactac gatcgggag  
5941 ggcttaccat ctggcccag tctgcaatg ataccgcgag acccagctc accggctcca  
6001 gattatcag caataacca gccagccgga agggccgagc gcagaagtgg tctgcaact  
6061 ttatccgct ccatccagtc tattaatgt tgcgggaag ctagagtaag tagttcgcca  
6121 gftaatagt tgcgcaactg tttgccatt gctacaggca tctgggtgc acgctcgtc  
6181 tttggtatg ctctatcag ctccggttcc caacgatcaa ggcgagttac atgatcccc  
6241 atgtgtgca aaaaagcgt tagctctc ggtctccga tctgtcag aagtaagtg  
6301 gccgcagtgt taccatcat ggtatggca gcaactgata attctctac tgtcatgcca

**FIG. 27C**

6361 tccgtaagat gcttttctgt gactggtag tactcaacca agtcattctg agaatagtgt  
6421 atgcggcgac cgagttgctc ttgccggcg tcaatacggg ataataccgc gccacatagc  
6481 agaactftaa aagtgtcat cattggaaaa cgttctcgg ggcgaaaact ctcaaggatc  
6541 ttaccgctgt tgagatccag ttcgatgtaa ccactcgtg cacccaactg atcttcagca  
6601 tctttactt tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa  
6661 aaggaataa gggcgacacg gaaatgtga atactcatic tcttccttt tcaatattat  
6721 tgaagcattt atcagggtta ttgtctcatg agcggataca tattgaaatg tatttagaaa  
6781 aataaacaaa taggggtcc gcgcacattt ccccgaaaag tgccacctga cgtc



FIG. 28

Vargula (463nm) and  
Red Italice (613nm)

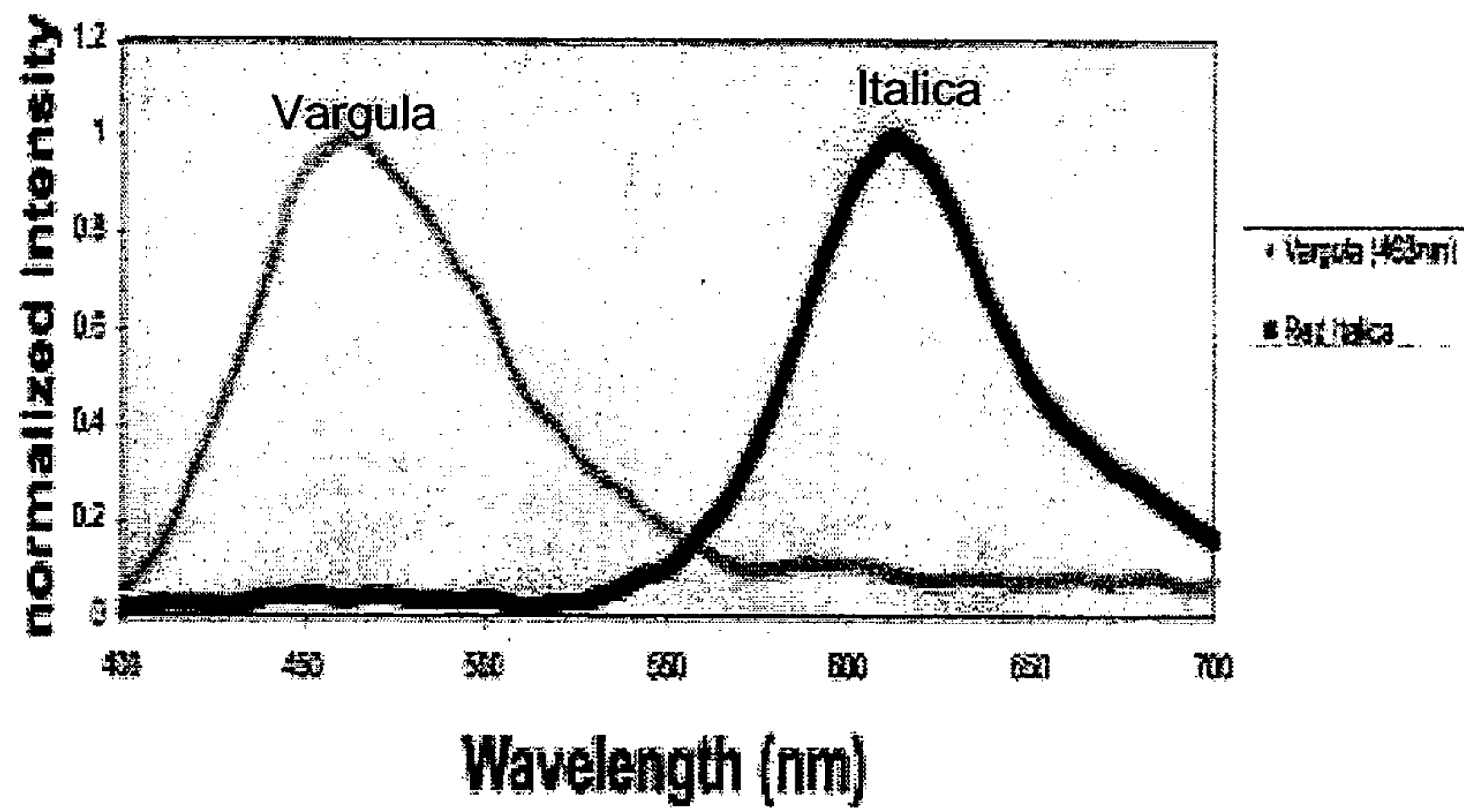


FIG. 29A

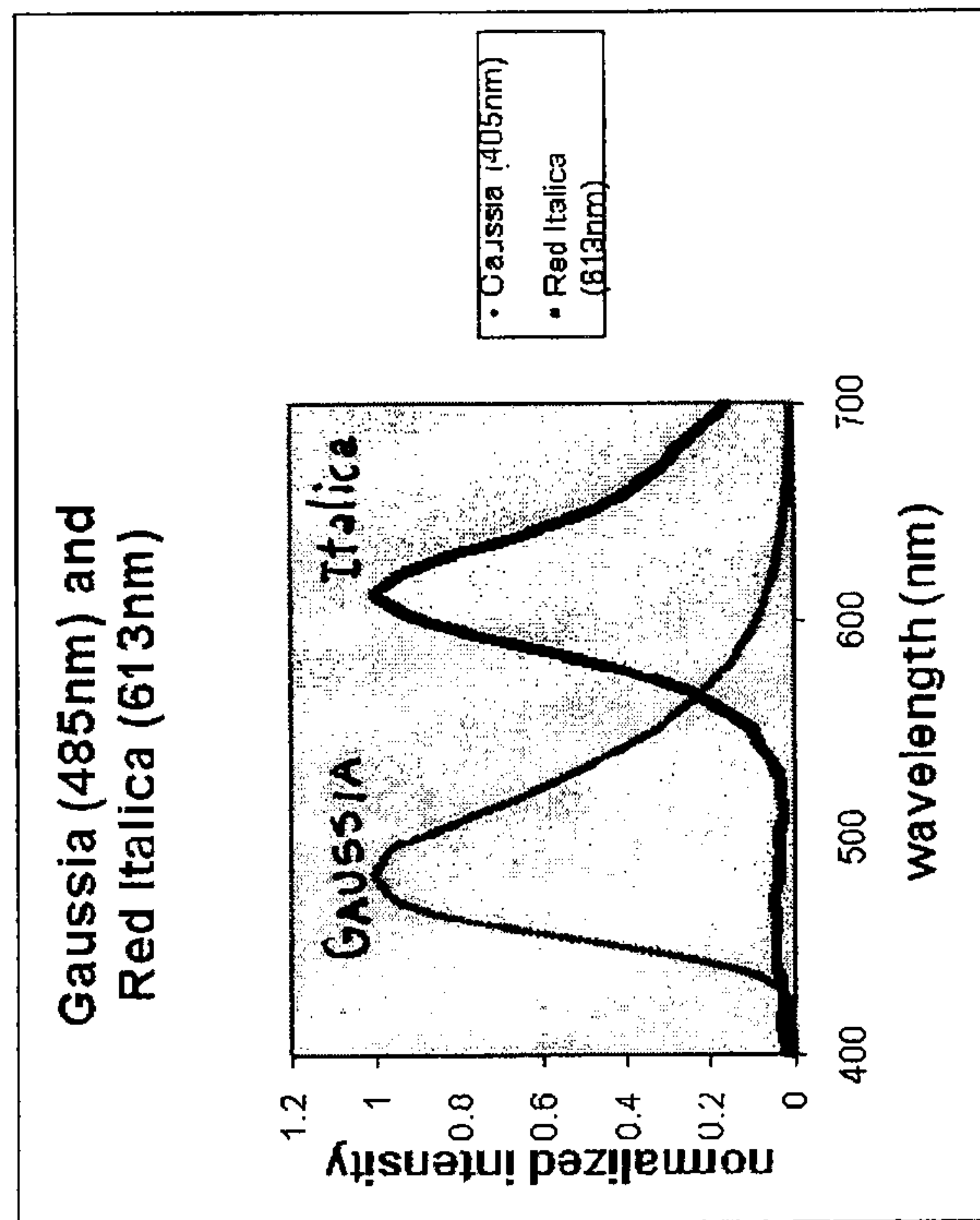


FIG. 29B

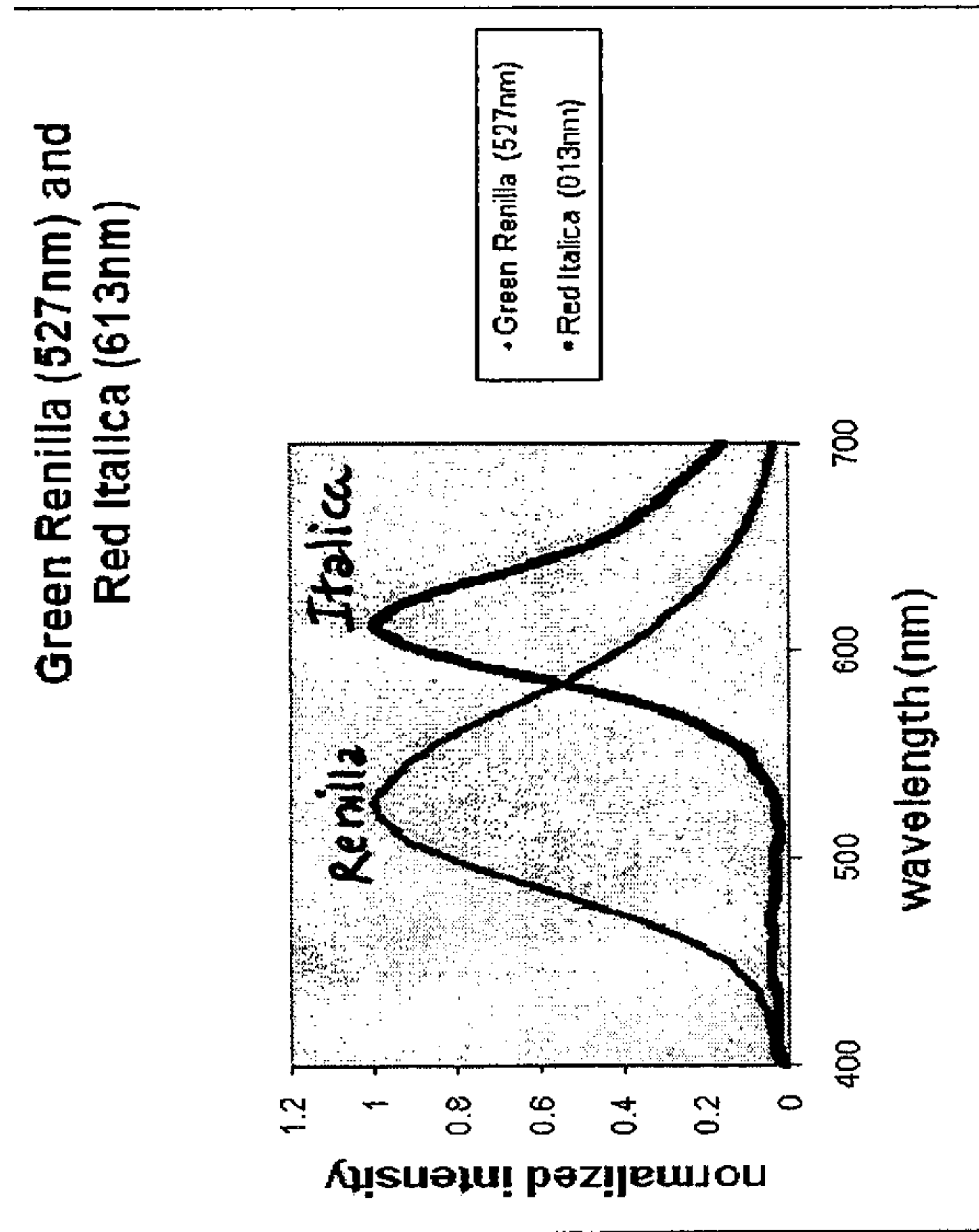


FIG. 30A

A mammalian expression vector expressing Red emitting firefly luciferase (human codon optimized signal) under control of the CMV promoter) (SEQ ID NO: 3)

Size: 6827 bases

```
1   gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
61  ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
121 cgagcaaaa ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181 ttagggtagt gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241 gattattgac tagtattaa tagtaataa ttacggggtc attagttcal agcccatata
301 tggagttcgg cgttacataa ctacggtaa atggcccgcc tggctgaccg cccaacgacc
361 cccgcccaat gacgtcaata atgacgatg tcccatagt aacgccaata gggactttcc
421 attgacgtca atgggtggac tatttacggt aaactgccc cttggcagta catcaaggtt
481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattatgca
601 tcgctattac catgggtgatg cggtttggc agtatacaaa tgggcgtgga tagcgggttg
661 actcacgggg attccaagt cccacccca ttgacgtcaa tgggagttg ttttggcacc
721 aaaatcaacg ggactttcca aaatgtcga acaactccgc cccattgacg caaatgggcg
781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca
841 ctgcttactg gcttatcga aataatacga ctactatag ggagacccaa gcttgggtacc
901 gagctcggat ccatggaaac agaaagagaa gaaaacgtt tctacggccc actgccattc
961 taccgatcgg aggggggctc tgcggcctc caattgcaca agtacatgca acaatagccc
1021 aagctcggcg ccatcgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081 tacttcgaca tcacgtgcag actcggcag gctatgaaga actacggcat gaagccagaa
1141 ggacacatcg ctctctgtag cgagaactgc gaagagtct tcaatcctgt tctggctggt
1201 cttacatcg gagttacagt cgcgccaact aacgaaatt atacacttag agagctgaac
1261 cacagtctgg ggatagccca acctactatc gtatctcta gcaggaaggc cctgcccaca
1321 gtgcttgagg tgcagaagac cgtgacttc atcaaaacca ttgtatcct ggacagtaag
1381 gtcaactcgc gcggttatga ctgcgtagag accttcaata agaaacacgt cgagctgggc
1441 tttcctgcca cctcattgt gccatcgac gtcaaagacc ggaagacca cattgtctg
1501 cttatgaact cttccggtc cacagggctg ccaaaggag tagagatcac tcacgaggcc
1561 ctggtcacga gattctcga cgttaaggac cctatatacg gcaatcaggt ggccccaggt
1621 accgctatcc tgactgtcgt gcctttccac cacggcttcg gaatgttca tctttgggc
1681 tactttgctc gcggttaccg gattgtcatg ctactaagt tcgacgagga gcttttctg
1741 cgcacacttc aggattaca gtgcactaca gtaactctgg tgcggacact gttcgcatt
1801 cttaataggt ctgagctcct tgataagtt gacctctcct acctgactga aatagccagc
1861 ggtgtgtgct cacttgccaa ggagatcggc gaggctgtg caagaagatt caacctcca
1921 ggcgtccggc agggatagtg actcaccgag actaccagt cctttatcat cactcctaag
1981 ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgtcaaggt gaagattatt
2041 gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101 ccatctccta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161 gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca ctctttata
2221 gtcgaccgcc tgaatctct cattaagat aaaggatacc aagtggcacc agctgaactg
2281 gactgtgtgc tctgcaaca cctaactat agagatcgt gttggccgg ggttccgac
2341 agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401 gagaaagaaa tctagacta tgtaaactcc caggtggica accacaagcg gctgaggggc
2461 ggcgtgcggt tctgatagta agtcccaag gggctcacag gaaagatcga cgcgaaagt
2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctgatctag aaataattct
2581 tactgtcatg ccaagtaaga tgcctttctg tctgcaata gcaggcatgc tggggatgcg
2641 gtggctcta tggcttctga ggcggaaga accagctggg gctctagggg gtatcccac
2701 gcgccctgta gggcgcatc aagcggcggc ggtgtggtgg ttacgcgacg cgtgaccgct
2761 acacttgcca gcgcctagc gccctctctc ttcgcttct tccctctt tctcggcag
2821 ttcgcccgtc tccccgta agctcctaat cggggcatcc ctttagggtt ccgatttagt
2881 gctttacggc acctcgacc caaaaaacti gattaggggt atggttcacg tagtgggcca
2941 tgcctctgat agacggttt tgcctcttg acgttgaggt ccacttctt taatagtga
3001 ctctgttcc aaactggaac aacctcaac cctactcgg tctattctt tgattataa
3061 gggattttgg ggatttcggc ctattggtta aaaaatgagc tgatttaaca aaaatttaac
3121 gcgaattaat tctgtggaat gttgtcagt tagggtgtgg aaagtccca ggctccccag
3181 gcaggcagaa gtatgcaag catgcatctc aattatgac caaccaggtg tggaaagtcc
3241 ccaggctccc cagcaggcag aagtatgca agcatgcatc tcaattatg agcaaccata
3301 gtcccggccc taactcggc catcccggc ctaactcgc ccagttcgc ccatctcgg
```



FIG. 30B

3361 ccccatggct gactaatttt tttatttat gcagaggccg aggccgcctc tgcctctgag  
3421 ctattccaga agtagtgagg aggcttttt ggaggcctag gcttttcaa aaagctccc  
3481 ggagcttga tatccatttt cggatctgat caagagacag gatgaggatc gttcgcgat  
3541 attgaacaag atggattgca cgcaggttct ccggccgctt gggtggagag gctattcggc  
3601 tatgactggg cacaacagac aatcggtcgc tctgatccg ccgtgtccg gctgtcagcg  
3661 caggggccc cggttcttt tgcaagacc gacctgtccg gtccctgaa tgaactgcag  
3721 gacgaggcag cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc  
3781 gacgtgtca ctgaagcggg aagggactgg ctgctattgg gcgaagtgc ggggcaggat  
3841 ctctgtcat ctaccttgc tctgcccag aaagtatcca tcatggctga tgcaatgcgg  
3901 cggctgata cgttgatcc ggctacctgc ccattcgacc accaagcga acatcgcac  
3961 gagcgagcac gtactcggat ggaagccggt ctgtcgtac aggatgatct ggacgaagag  
4021 catcaggggc tcgcccagc cgaactgtc gccaggctca aggcgcgat gcccgacggc  
4081 gaggatctc tcgtgacca tggcgatgcc tcttgcga atatcatgtt ggaatggc  
4141 cgtttctg gattcatga ctgtggccgg ctgggtgtgg cggaccgta tcaggacata  
4201 gcgttggta cccgtgatat tgctgaagag ctggcggcg aatgggctga ccgttctc  
4261 gtctttacg gtatcggcg tccgattcg cagcgcacg ctttctatc ctttctgac  
4321 gagttctct gagcgggact ctggggctc aaatgaccga ccaagcgacg cccaacctgc  
4381 catcacgaga ttctgatcc accgccgct tcatgaaag gttggcttc ggaatcgtt  
4441 tccgggacgc cggctggatg atcctccagc gcggggatct catgctggag ttctcggc  
4501 accccaactt gttattgca gcttataatg gttacaaata aagcaatagc atcacaatt  
4561 tcacaaata agcattttt tcactgcatt ctagtgtgg ttgtccaaa ctcatcaatg  
4621 tatctatca tgctgtata ccgtcgacct ctactagag ctggcgtaa tcatggtcat  
4681 agctgttcc tgtgtgaaat tgtatccgc tcacaattcc acacaacata cgagccggaa  
4741 gcataaagt taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttc  
4801 gctcactgcc cgtttccag tcgggaaacc tgctgtcca gctgcattaa tgaatcggc  
4861 aacgcgggg gagagcggg ttgcgtattg ggcgctctc cgttctctc ctaactgact  
4921 cgtcgcgctc ggtcgttcg ctgcggcgag cggatcagc tcaactcaag gcgtaatac  
4981 ggttatccac agaatcagg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa  
5041 aggccaggaa ccgtaaaaag gccgcgttc tggcgtttt ccataggctc cggccccctg  
5101 acgagcatca caaaaatga cgtcaagtc agagggtggc aaaccgaca ggactataaa  
5161 gataccaggc gtttcccc tgaagctccc tctgtcgc tctgttccg accctgccg  
5221 ttaccggata cctgtccgc ttttccctt cgggaagcgt ggcgcttct caatgctc  
5281 gctgtagga tctcagttc gtgtaggtc tctgtcca gctgggctgt gtgcacgaac  
5341 cccccgtca gcccgaccg tgcgcttat ccgtaacta tctcttgag tccaaccgg  
5401 taagacacga ctatcgca ctggcagcag cactggtaa caggattagc agagcagggt  
5461 atgtaggcgg tgctacagag tcttgaagt ggtggcctaa ctacggctac actagaagga  
5521 cagtattgg tatctgcgt ctgctgaagc cagttacct cggaaaaaga gttgtagct  
5581 ctgtatccg caacaaacc accgctgga gcggtggtt tttgtttgc aagcagcaga  
5641 ttacgcgag aaaaaagga tctcaagaag atctttgat ctttctac gggctgacg  
5701 ctcaatgaa cgaaaactca cgttaaggga tttgtgcat gagattatca aaaaggatct  
5761 tcaactagat cctttaaat taaaaatgaa gtttaaatc aatctaaagt atatatgagt  
5821 aaacttggc tgacagttc caatgctaa tcaatgaggc acctatctc gcgatctgc  
5881 tattcgttc atccatagt cctgactcc ccgtcgtga gataactac atacgggagg  
5941 gcttaccatc tggccccagt gctgcaatga taccgcgaga cccagctca ccggtccag  
6001 atttatcagc aataaaccag ccagccggaa gggccgagcg cagaagtgt cctgcaact  
6061 tatccgctc catccagtct attaattgt gccgggaagc tagagtaagt agttccag  
6121 ttaatagtt gcgcaactt gttgccatt ctacaggcat cgtgggtca cgtcgtcgt  
6181 ttggtatgg tcaatcagc tccggttcc aacgatcaag gcgagttaca tgatccccca  
6241 tgtgtgcaa aaaagcgggt agctccttc gtcctccgat cgtgtcaga agtaagtgg  
6301 ccgcatggt atcactcatg gttatggcag cactgcataa tctcttact gcatgcat  
6361 ccgtaagatg ctttctgtg actggtgagt actcaacaa gtcattctga gaatagtga  
6421 tgcggcgacc gagttgctt tccccggc caatacggga taataccgc ccatagca  
6481 gaacttaaa agtgctcatc attgaaaac gttctcggg gcgaaaactc tcaaggatct  
6541 taccgctgt gagatccagt tcatgtaac ccactcgtc acccaactga tctcagcat  
6601 ctttacttt caccagcgt tctgggtgag caaaaacagg aaggcaaat gccgcaaaa  
6661 aggaataag ggcgacacgg aatgttga tactcact cttctttt caatattt  
6721 gaagcattt tcagggtat tctctatga gcgatacat attgaaatg atttagaaa  
6781 ataaacaat aggggttccg cgcacattc cccgaaaagt gccacctgac gtc



FIG. 31A

**Sequence and Features of pCMV GrFLUC Vector:**

**A mammalian expression vector expressing human codon optimized green firefly luciferase (*Luciola Italica*) under control of the CMV promoter (SEQ ID NO: 4)**

Size: 6827 bases

pCMV-GrFLuc (6827 bp)

CMV promoter bases: 209-863

Green emitting firefly luciferase gene: 907-2560

T7 promoter bases: 1827-1845

Polylinker bases: 1852-1870

SP6 promoter: 2576-2593

Synthetic polyadenylation site: 2560-2604

SV40 promoter bases: 3145-3480

SV40 origin of replication: bases: 3259-3344

Neomycin ORF : bases 3516- 4310

SV40 PolyA: bases 4365-4737

ColE1 origin: bases 3934-4607

Ampicillin ORF: bases 4752-5612

```

1  gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaate tgctctgatg
61  ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggctgct gagtagtgcg
121  cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
181  ttagggtag  gcgttttcgc ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
241  gattattgac tagtattaa tagtaatcaa ttacggggtc attagttcat agcccatata
301  tggagtccg  cgttacataa ctacggtaa atggcccgc tggctgaccg cccaacgacc
361  cccgcccatt gacgtcaata atgacgtatg ttccatagt aacgccaata gggactttcc
421  attgacgtca atgggtggac tatttacggt aaactgcca cttggcagta catcaaggtg
481  atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
541  atgcccagta catgacctta tgggacttcc ctacttgcca gtacatctac gtattagtca
601  tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
661  actcacgggg attccaagt ctccacccca ttgacgtcaa tgggagtttg tttggcacc
721  aaaatcaacg ggactttcca aaatgctgta acaactccgc cccattgacg caaatgggcg
781  gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca
841  ctgcttactg gcttatcga aaataacga ctactatag ggagacccaa gcttggtagc
901  gagctcggat ccatggaaac agaaagagaa gaaaacgttg tctacggccc actgccattc
961  tacccgatcg aggagggctc tgcggcacc caatgcaca agtacatgca acaatagccc
1021  aagctcggcg ccatcgctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag
1081  tacttcgaca tcacgtgcag actcgccgag gctatgaaga actacggcat gaagccagaa
1141  ggacacatcg ctctcttag cgagaactgc gaagagtct tcttctctgt tctggctggt
1201  ctttacatcg gagttacagt cgcgccaact aacgaaatt atacacttag agagctgaac
1261  cacagtctgg ggatagccca acctactatc gtattctcta gcaggaaagg cctgcccaaa
1321  gtgcttgagg tgcagaagac cgtgactgac atcaaaacca ttgtatcct ggacagtaag
1381  gtcaacttcg gcggttatga ctgcgtagag acctcatta agaaacacgt cgagctgggc
1441  tttctgcca cctcattgt gccatcgac gtcaagacc ggaagacca cattgctctg
1501  cttatgaact ctccgggtc cacagggtc cccaaaggag tagagatcac tcacgaggcc
1561  ctggtcacga gattctctca cgtaaggac cctatatacg gcaatcaggt ggccccaggt
1621  accgctatcc tgactgtcat cctttccac cagccttcg gaatgagcac tactttgggc
1681  tactttgctt gcggttaccg gattgtcatg cttactaagt tcgacgagga gcttttctg
1741  cgcacacttc aggattacaa gtgcactagc gtaatcctgg tgcgacact gttcgcaatt
1801  ctaaataggt ctgagctcct tgataagttt gaccttcta acctgactga aatagccagc
1861  ggtggtgctc cacttgccaa ggagatcggc gaggctgttg caagaagatt caacctcca
1921  ggcgtccggc agggatatgg actcaccgag actaccagtg ctttatcat cactcctaag
1981  ggcgacgaca agccgggagc cagcggcaag gtcgtgcttc tgtcaaggt gaagattatt
2041  gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga
2101  ccatctctta tgttgggata cacgaacaat cctgaagcca ccagagaaac tattgacgag
2161  gaaggctggc tgcacacggg tgacatcggg tactacgacg aggatgagca cttcttata
2221  gtcgaccgcc tgaatctct cattaagtat aaaggatacc aagtgccacc agctgaactg
2281  gactctgtgc tctgcaaca ccctaacatt agagatgctg gtgtggccgg ggttcccagc
2341  agcgaggcag gcgagctgcc tggagccgtc gttgtgatgg aaaagggaaa gacaatgact
2401  gagaaagaaa tcgtagacta tgtaactcc caggtgtgca accacaagcg gctgaggggc
2461  ggctgctggt tcgtagatga agtcccaag gggctcacag gaaagatcga cgcgaaagt

```



FIG. 31B

2521 atcagggaga tactcaagaa acctcaagca ggtgggtagt ctagaataa ttctactgt  
2581 catgccaagt aagatgcttt tctgtgctgc aatagcaggc atgctgggga tgcggtgggc  
2641 tctatggctt ctgaggcggga aagaaccagc tggggctcta gggggtatcc ccacgcgcc  
2701 tgtagcggcg cattaagcgc ggcgggtgtg gtggttacgc gcagcgtgac cgctacactt  
2761 gccagcgccc tagcggccgc tccittcgtt ttctccctt ccttctcgc cacgttcgcc  
2821 ggctttcccc gtcaagctct aatcggggc atcccttag ggttccgatt tagtgctta  
2881 cggcacctcg acccaaaaa actigattag ggtgatggtt cacgtagtgg gccatcgccc  
2941 tgatagacgg ttttcgccc ttgacgttg gagtccactg tcttaatag tggactctg  
3001 ttccaaactg gaacaact caacctatc tgggtctatt ctttgattt ataagggtt  
3061 ttggggattt cggcctattg gttaaaaaat gagctgattt acaaaaaatt taacgcgaat  
3121 taattctgtg gaatgtgtgt cagttagggt gtggaaagtc cccaggctcc ccaggcaggc  
3181 agaagtatgc aaagcatgca tctcaattag tcagcaacca ggtgtggaaa gtccccaggc  
3241 tccccagcag gcagaagat gcaaagcatg catctcaatt agtcagcaac catagtcccg  
3301 ccctaactc cgccatccc gccctaact cggccagtt cggccattc tccgcccatt  
3361 ggctgactaa tttttttat ttatgcagag gccgaggccg cctctgcctc tgactatc  
3421 cagaagtagt gaggaggctt tttggaggc ctaggctttt gcaaaaagct cccgggagct  
3481 tgtatatcca tttcggatc tgatcaagag acaggatgag gatcgtttcg catgattgaa  
3541 caagatgatg tgcacgcagg ttcccgccg gcttgggtgg agaggctatt cggctatgac  
3601 tgggcacaac agacaatcgg ctgctctgat gccgccgtgt tccggctgtc agcgcagggg  
3661 cgcccggtc ttttgtcaa gaccgacctg tccggtgccc tgaatgaact gcaggacgag  
3721 gcagcgggc tctgtggct ggccacgacg ggcgttcctt gcgagctgt gctcagctt  
3781 gtcactgaag cgggaaggga ctggctgcta tgggcgaag tccggggca ggtctcctg  
3841 tcatctacc ttgctcctgc cgagaaagta tccatcatgg ctgatgcaat gcggcggctg  
3901 catacgctt atccggctac ctgcccattc gaccaccaag cgaacatcg catcagcga  
3961 gcagctactc ggatggaagc cggctctgtc gatcaggatg atctggacga agagcatcag  
4021 gggctcgcgc cagccgaact gttcggcagg ctcaaggcgc gcattgcccga cggcgaggat  
4081 ctctctgta cccatggcga tgcctgctg ccgaatatca tggtggaata tggccgctt  
4141 tctggattca tgcactgtgg ccggctgggt gtggcggacc gctatcagga catagcgtt  
4201 gctaccctg atattgctga agagcttggc ggcgaatggg ctgaccgctt cctcgtgctt  
4261 tacggatcg ccgctcccga ttcgacgagc atcgccttct atcgccttct tgacgagtc  
4321 ttctgagcgg gactctgggg ttgaaatga ccgaccaagc gacgccaac ctgcatcac  
4381 gagattcga ttccaccgcc gccttctatg aaagggtggg ctccggaatc gtttccggg  
4441 acgcccggctg gatgatctc cagcgcgggg atctcatgct ggagtctc gccacccca  
4501 acttcttat tgcagctat aatggttaca aataaagcaa tagcatcaca aattcaca  
4561 ataaagcatt ttttactg cattctagt gtggtttgc caaactcacc aatgtaact  
4621 atcatgtctg taccctgc acctctagct agagcttggc gtaatcatgg tcatagctgt  
4681 ttctgtgtg aaattgttat ccgctcaca ttccacaca catacagacc ggaagcataa  
4741 agttaaagc ctgggtgccc taatgagtg gtaactcac ataatgctg ttgcgctcac  
4801 tccccgctt ccagtcggga aacctgctg gccagctgca ttaatgaatc ggccaacgcg  
4861 cggggagagg cggtttgcgt attggcgcct ctccgctc ctgctcact gactcgtgc  
4921 gctcggctgt tggctgccc cgagcggat cagctcactc aaaggcggia atacggtat  
4981 ccacagaatc aggggataac gcaggaaaga acatgtgagc aaaaggccag caaaaggcca  
5041 ggaaccgtaa aaaggccgcg ttgctggcgt tttccatag gctccgccc cctgacgagc  
5101 atcaaaaaa tgcagctca agtcagaggt ggcgaaacc gacaggacta taaagatacc  
5161 aggcgttcc cctggaagc tccctcgtc gctctcctgt tccgacctg ccgcttaccg  
5221 gatacctgc cgcttctc cctcgggaa cgttggcgtt ttctaatgc taccgctgta  
5281 ggtatctcag ttcggtgtag gtcgttcgct ccaagctggg ctgtgtgac gaacccccg  
5341 ttacggcga ccgctgccc ttatccgta actatcgtct tgagtccaac ccgtaagac  
5401 acgactatc gccactgca gcagccactg gtaacaggat tagcagagcg aggtatgtag  
5461 gcggtgctac agagtcttg aagtgtggc ctaactacgg ctacactaga aggacagat  
5521 ttggtatctg cgtctgctg aagccagta ccttcggaaa aagagtgtgt agctcttgat  
5581 ccggcaaca aaccaccgt ggtagcggg gttttttgt ttgcaagcag cagattacgc  
5641 gcagaaaaa aggatctca gaagatcct tcatctttc tacggggtct gacgctcagt  
5701 ggaacgaaa ctacgtaaa gggatttgg tcatgagatt atcaaaaagg atctcacct  
5761 agatcctttt aattaaaaa tgaagtitta aatcaacta aagtataat gagtaactt  
5821 ggtctgacag ttaccaatgc ttaactagtg aggcacctat ctacagcgtc tctctatctc  
5881 gttcatccat agttgctga ctcccgtcg ttagataac tacgatacgg gagggcttac  
5941 catctggccc cagtgtgca atgataccgc gagaccacg ctaccggct ccagattat  
6001 cagcaataaa ccagccagcc ggaaggccg agcgcagaag tggctctgca actttatccg  
6061 cctccatca gtctattaat tgtgcccgg aagctagagt aagtgtctg ccagtaata  
6121 gttgcaaa cgttgttgc attgctacag gcactgtgt gtcacgctc tcttttgta



## FIG. 31C

6181 tggcttcatt cagctccggt tcccaacgat caaggcgagt tacatgatcc cccatgttgt  
6241 gcaaaaaagc ggtagctcc ttcggctctc cgatcggtgt cagaagtaag ttggccgcag  
6301 tggatcact catggtatg gcagcactgc ataattctct tactgtcatg ccatccgtaa  
6361 gatgctttc tgtgactggt gactactcaa ccaagtcatt ctgagaatag tgtatgcggc  
6421 gaccgagttg ctctgcccg gcgcaatac gggataatac cgcgccacat agcagaactt  
6481 taaaagtgct catcattgga aaacgttctt cggggcgaaa actctcaagg atctaccgc  
6541 tgttgagatc cagttcgatg taaccactc gtgcacccaa ctgatcttca gcactttta  
6601 cttcaccag cgttctggg tgagcaaaaa caggaaggca aaatgccgca aaaaaggaa  
6661 taaggcgac acggaaatgt tgaatactca tactcttct tttcaatat tattgaagca  
6721 ttatcaggg ttattgtctc atgagcggat acatattga atgtattag aaaaataaac  
6781 aaataggggt tccgcgcaca ttccccgaa aagtccacc tgacgtc

FIG. 32A

The sequence of the CMV expression vector expressing human codon optimized Vargula luciferase under control of the CMV promoter (not the vargula luciferase sequence) is in bold.

CMV promoter bases: 209-863  
 Vargula luciferase gene: 907-  
 T7 promoter bases: 864-882  
 Polylinker bases: 889-907

gacggatcgggagatctcccgatccctatggctgactctcagtaacaatc tgctctgatgccgcatagtaagccagatctgctccctgcttgtgtgt  
 ggaggtcgtgtagtgctgcgagcaaaattaaagctacaacaaggcaag gcttgaccgacaattgcatgaagaatctgcttagggtaggcgtttgctg  
 ctgcttcgcatgtacggccagatatacgcgttgacattgattattgac tagttattaatagtaatacaattacggggtcattagttcatagcccatata  
 tggagttccgcgttacataacttacggtaaatggccgcctggctgaccg cccaacgacccccgccattgacgtcaataatgacgtatgttcccatagt  
 aacgccaatagggactttccattgacgtcaatgggtggactattacggg aaactgccactggcagtcacatcaagtgtatcatatgccaagtacgccc  
 cctattgacgtcaatgacggtaaatggccgcctggcattatgccagta catgacctatgggactttcctacttggcagtcacatctacgtattagta  
 tcgctattaccatgggtgatgcggtttggcagtcacatcaatgggcgtgga tagcggttgactcacggggattccaagtctccacccattgacgtcaa  
 tgggagttgtttggcaccaaaatcaacgggactttcaaaatgtgta acaactccgccccattgacgcaaatggcggttaggcgtgtacgggtgggag  
 gtctatataagcagagctctctggtaactagagaacccactgcttactg gcttatcgaaataatagactcactatagggagaccaagcttgggtacc  
 gagctc **ATGAAGATAATTATCCTTTCTGTGATTCTGGCTTACTGTGTTAC**  
**AGTGAATTGTCAGGATGCATGTCCAGTAGAGGCGGAACCGCCATCTTCTA**  
**CCCCGACCGTACCAACCTCCTGCGAAGCTAAAGAAGGGGAGTGCATCGAT**  
**ACAAGGTGCGCTACCTGCAAACGGGATATCCTGTCCGACGGACTTTGCGA**  
**AAATAAACCCGGAAGACCTGCTGTGCAATGTGTGTCAGTATGTCATCGAAT**  
**GCCGGTTCGAGGCCCGCGTTATTTAGAACATTTTACGGTAAACGGTTT**  
**AATTTCCAGGAACCCGGCAAATACGTAAGTGGCTCGCGGCACCAAGGGTGG**  
**CGACTGGAGCGTCACCCTGACAATGGAAAACCTGGACGGGCAGAAAGGAG**  
**CCGTGCTTACTAAACTACCTGGAGGTGGCGGGAGACGTAATTGACATC**  
**ACTCAGGCAACGGCTGACCCAATAACCGTGAACGGAGGAGCTGATCCCGT**  
**GATTGCAAACCTTTCACTATTGGCGAGGTCACGATTGCCGTCGTCGAAA**  
**TTCCAGGCTTCAACATCACAGTGATCGAGTTCCTCAAGCTGATCGTCATT**  
**GATATCCTCGGCGGACGGTCCGTTCCGCATCGCACCTGACACAGCCAACAA**  
**GGGCTGATCTCTGGCATTGTTGGTAACCTTGAAATGAATGATGCTGATG**  
**ACTTCACAACGGACGCCGACCAACTGGCCATTCAACCTAATATCAACAAA**  
**GAGTTTGATGGATGTCCCTTTACGGAAATCCTTCAGACATCGAATACTG**  
**CAAAGGCCTCATGGAACCGTACCGGGCCGTTTGCAGAAATAACATCAACT**  
**TCTACTATTATACTCTGAGCTGCGCATTGTCATACTGTATGGGCGGTGAG**  
**GAGAGAGCCAAACATGTGCTTTTCGACTATGTGGAGACCTGCGCCGCCCC**  
**GGAGACTCGCGGTACCTGCGTCCTGAGCGGCCATACCTTCTATGACACCT**  
**TCGATAAGGCTAGGTACCAGTTCGAAGGGCCTTGCAAAGAGCTCCTGATG**  
**GCCGCAGATTGTTACTGGAACACTTGGGACGTCAAAGTTTCCCATCGGGA**  
**CGTAGAGAGCTACACGGAAGTTGAGAAGGTGACCATCAGGAAGCAGAGTA**  
**CCGTGCTAGACCTGATCGTCGACGGCAAGCAGGTAAAGGTAGGAGGCGTG**  
**GACGTTAGTATTCCGTATTCTTCTGAAAATAACGAGCATCTACTGGCAGGA**  
**TGGAGACATTCTGACAACCGCCATCCTTCCAGAAGCTCTGGTGGTGAAGT**  
**TTAACTTCAAGCAGCTGCTGGTAGTGACATTGCGGACCCATTGACGGG**  
**AAAACCTGTGGGATTTGCGGCAACTACAACCAGGACTCAACTGACGATTT**  
**CTTTGACGCCGAAGGGGCTTGCCTCTTACCCCAAATCCGCCTGGATGCA**  
**CCGAAGAGCAAAGCCTGAAGCGGAACGGCTGTGCAATTCAGTGTGAT**  
**TCTTCAATAGATGAGAAATGCAACGTGTGTTACAAACCTGACCGCATCGC**  
**ACGCTGCATGTATGAGTATTGCCTGAGAGGTCAACAAGGGTTCTGCGATC**  
**ACGCGTGGGAATTTAAGAAAGAATGCTACATAAAGCACGGGGATACATTG**  
**GAGGTGCCGCCAGAATGCCAGTAGTctagaaataaacttactgtcatgccaagtaagatgcttttctgtgctgcaat**  
 agcaggcatgctgggatcggtggctctatggcttctgagcggaag aaccagctgggcttaggggtatccccacgcccctgtagcggcgc  
 taagcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgc agcgccttagcggcctctcttctgctttcttcccttcttctgcccac  
 gttcggcggctttccccgtcaagcttaaatggggcatcccttaggggt tccgatttagtctttacggcacctcgaccccaaaaaacttgattagggt  
 gatgggtcacgtatggccatcgcctgatagacggttttgccttt gacgttgagtcacgttcttaatagtgactctgttccaaactggaa  
 caaactcaacctatctcggctattctttgattataagggattttg gggatttcggcctattggttaaaaaatgagctgatttaacaaaaattaa  
 cgggaatfaattctgtggaatgtgtgctaggtgtgaaagtcccc aggtccccaggcaggcagaagtatgcaagcatgcatctcaattagtca  
 gcaaccaggtgtgaaagtccccaggctccccagcaggcagaagtatgca aagcatgcatctcaattagtcagcaacctagtcggcccccttaactcgc  
 ccatcccccttaactcggccagttccgcccatttccgcccattggc tgactaattttttatfatgagaggccaggccgcctctgctctga  
 gctattccagaagtagtgaggagctttttggaggcctagcctttgca aaaagctccccggagctgtatatccatttccgatctgatcaagagaca  
 ggataggatcgtttcgcattgattgaacaagatggattgcacgcagggtc tccggccctgggtggagaggctattcggctatgactgggcacaacaga



FIG. 32B

caatcggctgctctgatgccccgtgtccggctgtcagcgcaggggccc cgggtctttttgtcaagaccgacctgtccgggtgccctgaatgaactgca  
ggacgaggcagcgcggctatcgtggctggccacgacgggcttccctgcg cagctgtgctcgcaggtgtcactgaagcgggaaggactggctgctattg  
ggcgaagtccggggcaggatctcctgtatctcacctgtcctgcccga gaaagtatccatcatggctgatgcaatcggcggtgcatacgtgatc  
cggctacctgccattcgaccaccaagcgaacatcgcacgagcgagca cgtactgggatggaagccggtcttctgatcaggatgatctggacgaaga  
gcatcaggggctcgcgccagccgaactgtccagggctcaaggcgcgca tgcccagcggcaggatctcgtcgtgacctggcgatgctgcttggcc  
aatatcatggtggaaaatggccgcttttctgattcatcagctgtggccg gctgggtgtggcggaccgtatcaggacatagcgttggctaccctgata  
ttgctgaagagcttggcggcgaatgggctgaccgcttctcgtgctttac ggtatcggcctcccattcgcagcgcacgccttctatgccttctga  
cgagttctctgagcgggactctggggctcgaatgaccgaccaagcgac gcccaacctgccatcacgagattcgaattccaccgcccttctatgaaa  
ggttgggcttcggaatcgtttccgggacgccggctggatgatctccag cgcggggatctatgctggagttctcggccaccccaactgtttattgc  
agcttataatggttacaataaagcaatagcatcacaatttcacaaata aagcattttttcactgcattctagtgtgtgttgcacaaactcatcaat  
gtatctatcatgtctgtatacctgcacctctagctagagcttggcgta atcatggtcatagctgttctgtgtgaaattgtatccgctcacaattc  
cacacaacatacagaccggaagcataaagtgtaaagcctggggtgcctaa tgagttagtaactcacattaattgcgttgcgctcactgccgcttcca  
gtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcgg ggagaggcgggttgcgtattggcgctcttccgcttctcgtcactgac  
tcgctcgcctcggctcgtcggctcggcgagcggatcagctcactcaaa ggcggtaatacgggttatccacagaatcaggggataacgcaggaaagaaca  
tgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcttg ctggcgttttccataggctccgccccctgacgagcatcaaaaaatcg  
acgctcaagtcagaggtggcgaacccgacaggactataaagataccagg cgtttccccctggaagctccctcgtcgcctcctctgtccgacctgccg  
cttaccggatacctgtccgcttctccttccgggaagcgtggcgcttcc tcaatgctcacgctgtaggtatctcagttcgggtgtagctcgtcctcca  
agctgggctgtgtgcacgaacccccgtcagcccagcgtcgcctta tccgtaactatcgtctgagtcacaacccggaagacacgacttatgcc  
actggcagcagccactgtaacaggattagcagagcaggtatgtaggcg gtgtacagagttcttgaagtgggtggcctaactacggctacactagaagg  
acagtattggtatctgcgctctgctgaagccagttaccttcggaaaaag agttggtagctctgatccggcaacaaaccaccgctggtagcgggtgt  
ttttgtttgcaagcagcagattacgcgcagaaaaaaggatctcaagaa gatcctttgatctttctacggggtctgacgctcagtggaacgaaaactc  
acgttaagggttttggatgatgattatcaaaaaggatcttcacctaga tcttttaaaatlaaaaatgaagttttaaataatctaaagtatatatgag  
taaacttggctgtacagttaccaatgcttaatcagtgaggcacctatctc agcgatctgtctattcgttcatccatagttgcctgactcccgtcgtgt  
agataactacgatacgggagggttaccatctggcccagtgctgcaatg ataccgcgagaccacgctcaccggctccagatttatcagcaataaacca  
gccagccggaaggccgagcgcagaagtgtcctgcaactttatccgct ccatccagctatfaattgttggcgggaagctagagtaagtagttcgcca  
gtaatagtttgcgcaacgttgttgcattgctacaggcatcgtgggtgc acgctcgtcgttggatggctcattcagctccgggtcccaacgatcaa  
ggcgagttacatgatccccatgtgtgcaaaaaagcgggttagctcctc ggtcctccgacgtgtgcagaagtaagtggccgcagttatcactcat  
ggttatggcagcactgcataattcttactgtcatgccatccgtaagat gctttctgtgactggtgagtactcaaccaagtcattctgagaatagtg  
atgcggcgaccgagttgcttggccggcgtcaatacgggataataccgc gccacatagcagaacttfaaaagtctcatcattgaaaacgttctcgg  
ggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaa cccactcgtgcacccaactgatcttcagcatctttactttaccagcgt  
ttctgggtgagcaaaaacaggaaggcaaatgccgcaaaaaagggaataa gggcgacacggaaatgttgaatactcactcttcttttcaatattat  
tgaagcatttatcagggttattgtctcatgagcggatacatattgaaatg taittagaaaaataaacaataggggttccgpcacatttcccgaag  
tgccacctgacgtc



# MULTIPLEX ASSAYS WITH MULTIPLE LUCIFERASES REPORTERS AND USES THEREOF

## RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/238,146, filed on Aug. 29, 2009, which is hereby incorporated by reference in its entirety.

## FIELD OF THE INVENTION

The present invention concerns the field of luciferase reporters useful in biological and biochemical assays.

## BACKGROUND OF THE INVENTION

Luciferases are enzymes that catalyze reactions that emit light. Luciferases are named according to their source organisms such as beetles (firefly) or marine organisms. Examples of bioluminescent marine animals include: *Renilla*, also known as sea pansies, which belong to a class of coelenterates known as the anthozoans. In addition to *Renilla*, other representative bioluminescent genera of the class Anthozoa include *Cavarnularia*, *Ptilosarcus*, *Stylatula*, *Acanthoptilum*, and *Parazoanthus*. All of these organisms are bioluminescent and emit light as a result of the action of an enzyme (luciferase) on a substrate (luciferin) under appropriate biological conditions.

Different luciferases have different properties with regard to substrate specificity and intensity of light emission and stability of the bioluminescent signal, which is commonly measured by a luminometer. Luciferases are useful as transcriptional reporter genes and in imaging reporter gene expression in living subjects and many other applications in molecular biology.

Certain luciferases, such as those that utilize *cypridina* luciferin (vargulin) as a substrate, can be useful reporters because of their strong luminescent signal and the fact that they are secreted in the native form. However *cypridina* luciferin (vargulin) is very difficult to synthesize (usually involving an 18-step chemical synthesis). The limiting supply and the cost of the material have made the assay difficult to commercialize.

## SUMMARY OF THE INVENTION

Accordingly, the present invention provides modified luciferases, methods of making modified luciferases, and methods of using modified luciferases.

In one aspect, the present invention provides an isolated polynucleotide that encodes a modified *Luciola Italica* (also referred to as *L. Italica*) luciferase. In a further aspect, the modified *L. Italica* luciferase shows increased luciferase activity when expressed in mammalian cells as compared to a non human codon optimized mutant *L. Italica* luciferase.

In an embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that shows an approximately 1000-fold increased luciferase activity when expressed in mammalian cells as compared to a non human codon optimized mutant *L. Italica* luciferase.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is a red-emitting luciferase with an emission maximum of approximately 617 nm.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is human codon-optimized.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that is a green-emitting luciferase with an emission maximum of approximately 550 nm.

In a further embodiment and in accordance with any of the above, the present invention provides a modified *L. Italica* luciferase that includes a secretory signal at its amino terminal end. In a still further embodiment, the secretory signal is a chymotrypsinogen secretory signal.

In one aspect, the present invention provides assays utilizing any of the modified luciferases discussed herein. In a further aspect, the assays are multiplexed reporter assays.

In one aspect, the present invention provides an isolated polynucleotide that encodes a modified *Renilla* luciferase. In a further aspect, the modified *Renilla* luciferase shows increased activity and stability over a native human codon optimized *Renilla* luciferase.

In an exemplary embodiment the present invention provides a modified *Renilla* luciferase that is a green-emitting *Renilla* luciferase.

In a further embodiment and in accordance with any of the above, the invention provides a modified *Renilla* luciferase that includes a secretory signal at its amino terminal end.

In one aspect, the present invention provides multiplexed luciferase assays comprising at least two different luciferase reports, where the at least two different luciferase reporters emit at two different wavelengths and/or utilize different substrates.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows data for relative luciferase stability for a *Cypridina* assay conducted using reagents without sodium chloride (VLAR-1) and with sodium chloride (VLAR-1 with sodium chloride).

FIG. 2 shows data for time course of activity in a *Cypridina* assay using 5  $\mu$ l of sample (FIG. 2A) or 20  $\mu$ l of sample (FIG. 2A).

FIG. 3A-B shows the sequence of a green *Renilla* luciferase plasmid (SEQ ID NO: 1).

FIG. 4 shows the sequence of a modified red firefly luciferase with a secretory signal (SEQ ID NO: 2).

FIG. 5 shows data from a *Cypridina* luciferase assay in varying concentrations of sodium chloride.

FIG. 6 shows data from a *Renilla* luciferase assay with and without stabilizer (NP40).

FIG. 7 shows data comparing luciferase activity of native human codon optimized *Renilla* luciferase and a mutant *Renilla* luciferase of the invention.

FIG. 8 shows data comparing luciferase activity of human codon optimized and non-human codon optimized red-emitting *L. Italica* luciferase.

FIG. 9 shows data comparing luciferase activity of human codon optimized and non-human codon optimized green-emitting *L. Italica* luciferase.

FIG. 10 shows data comparing luciferase activity of intracellular red-emitting *L. Italica* luciferase and secreted red-emitting *L. Italica* luciferase.

FIG. 11 shows data comparing luciferase activity of secreted red *L. Italica* luciferase in the lysate and the supernatant from HEK293 cells.

FIG. 12 shows kinetics of luciferase activity in (A) Red *Luciola* luciferase, (B) *Guassia* luciferase, (C) *Cypridina* luciferase, and (D) Green *Renilla* luciferase.



FIG. 13 shows emission spectra from (A) a double reporter assay with *Vargula* and Red *Italica* luciferases and (B) a triple reporter assay with *Vargula*, Green *Renilla* and Red *Italica* Luciferases.

FIG. 14 shows kinetics data of a *Gaussia* luciferase assay using a GAR-1 reagent.

FIG. 15 shows data comparing stabilities of *Gaussia* luciferase assays using the GAR-2 reagent are in the presence of a stabilizer (FIG. 15A) and in the absence of a stabilizer (FIG. 15B).

FIG. 16 shows data related to relative luciferase activity of a firefly luciferase assay.

FIG. 17 shows data of relative luciferase activity of a *Cypridina* luciferase assay.

FIG. 18 shows data comparing luciferase activity of a modified *Vargula* luciferase of the invention in the lysate and the supernatant from mammalian cells.

FIG. 19 shows kinetic data for luciferase activity in a *Cypridina* luciferase assay.

FIG. 20 shows data comparing relative luciferase activity of Green *Renilla* luciferase in the absence (top panel) and presence (bottom) of a stabilizer.

FIG. 21 shows data from a firefly luciferase assay in the presence (square) and absence (diamonds) of a stabilizer.

FIG. 22 shows data from a dual assay of the invention utilizing firefly and *Cypridina* luciferases.

FIG. 23 shows data from a dual assay of the invention utilizing *Cypridina* (Panel A) and *Renilla* (Panel B) luciferases.

FIG. 24 shows emission spectra from a dual assay of the invention utilizing *Vargula* and Green *Renilla* luciferases.

FIG. 25 shows data from a triple assay of the invention utilizing *Cypridina*, firefly and *Gaussia* luciferases.

FIG. 26 shows emission spectra from a triple assay of the invention utilizing *Cypridina*, Green *Renilla* and Red *Italica* luciferases.

FIG. 27 shows the sequence of a red firefly luciferase of the invention (SEQ ID NO: 5).

FIG. 28 shows emission spectra from a dual assay of the invention utilizing *Vargula* and Red *Italica* luciferases.

FIG. 29 shows emission spectra from a dual assay of the invention utilizing (A) *Gaussia* and Red *Italica* luciferases and (B) Green *Renilla* and Red *Italica* luciferases.

FIG. 30 shows the sequence of a red emitting firefly human codon optimized luciferase of the invention (SEQ ID NO: 3).

FIG. 31 shows the sequence of a human codon optimized green firefly luciferase of the invention (SEQ ID NO: 4).

FIG. 32 shows the sequence of a human codon optimized *Vargula* luciferase of the invention (SEQ ID NO: 6).

#### DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing devices, formulations and methodologies which are described in the publication and which might be used in connection with the presently described invention.

Note that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a polymerase” refers to one agent or mixtures of such agents, and reference to “the method”

includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention. It will be apparent to one of skill in the art that these additional features are also encompassed by the present invention.

#### Overview

The present invention provides modified luciferases and/or combinations of luciferases, and methods of utilizing those luciferases in reporter gene assays. In addition, the invention provides reagents that provide increased stability and activity in assays using luciferase reporters.

The present invention provides modified (also referred to herein as “mutant” or “variant”) luciferases showing improved activity over wildtype luciferases or other modified luciferases known in the art reported to have improved properties for reporter gene assays or in vivo imaging applications. As used herein, “wildtype luciferases” refers to any luciferase that occurs in nature.

In certain aspects, the present invention provides modified luciferases that show brighter luminescence when expressed in mammalian cells as compared to the luminescence seen when wildtype luciferases are expressed in mammalian cells. The present invention also provides a method of expressing luciferase as a very bright intracellular reporter (not secreted) by sequence modification to increase its utility as an intracellular reporter in multiplexed assays and for imaging applications. The present invention also provides a composition for assays utilizing luciferases that lowers the cost and increases the efficiency and sensitivity of the assay by altering the reaction conditions such that high luminescence is produced using significantly less amount of luciferin.

The present invention further provides reagents for assays utilizing modified luciferases of the invention as well as mammalian expression vectors expressing secreted and intracellular luciferases.

In further embodiments the present invention provides sequence modifications (human codon optimization) to nucleotides encoding luciferases which result in an approximately 1000-fold increase in luciferase expression in transfected mammalian cells compared to the non-human codon optimized versions of these genes.

In further embodiments, the invention provides novel secreted reporter modified luciferases that are about 5 to about 35 fold brighter than wildtype luciferases. Such luciferases are used in accordance with the present invention as stand alone reporters or in multiplexed luciferase assays in combination with one or more other luciferases. As will be appreciated, combinations of luciferases for multiplexed assays of the invention can include both wildtype and modified luciferases.



In further aspects, the present invention provides assay compositions for measurement of modified luciferases of the invention as single luciferase assay formats. In further aspects of the invention, assay compositions are provided that enable simultaneous measurement of at least two different reporters in cell lysates or supernatants using a single assay solution. The luciferase activities of multiple reporters are analyzed by exploiting spectral differences in the emission maxima of the different luciferases.

Improved luciferases used in the present invention include without limitation: (i) a red-emitting firefly luciferase (Red-Fluc) from the Italian firefly *Luciola Italica* (emission max 609 nm), including intracellular (non-secreted) variants and secreted variants generated by fusing a chymotrypsinogen secretory signal sequence to the amino terminal end of the luciferase; (ii) a green-emitting firefly luciferase (Green-Fluc) from the Italian firefly *Luciola Italica* (emission max 550 nm), including intracellular (non-secreted) variants and secreted variants generated by fusing a chymotrypsinogen secretory signal sequence to the amino terminal end of the luciferase; (iii) a *Cypridina* Luciferase or *Vargula* luciferase (VLuc) from the marine ostracod *Vargula Hilgendorfi*, a secreted luciferase (emission max 395 nm or 462 nm depending on the substrate used); (iv) *Vargula* luciferase that has been modified at the C-terminal end with a KDEL sequence (endoplasmic reticulum retention signal) so that it is expressed intracellularly-VLuc-KDEL; (v) a modified secreted blue-emitting (emission max 480 nm) *Renilla* luciferase (B-Rluc) which is brighter and more stable than native *renilla reniformis* luciferase; (vi) a green emitting secreted *Renilla* luciferase (emission max 535 nm) modified to be secreted by fusing a synthetic secretory signal encoding gene sequence in frame with the gene encoding the green emitting modified of *renilla* luciferase; (vii) a *Gaussia* luciferase (emission max 482 nm) either native secreted (Gluc) or modified to be expressed intracellularly (Gluc-KDEL).

#### Luciferases of the Invention

Modified luciferases of the present invention show increased signal magnitude and stability. In certain embodiments, modified luciferases of the invention show at least a 1, 2, 3, 4, 5, 10, 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000-fold increase in the magnitude of the signal over signals seen with wildtype luciferases.

Modified luciferases of the invention may be intracellular (i.e., not secreted), or they may be modified to be secreted. In further embodiments, modified luciferases of the invention are engineered to further express a secretory signal, general at the amino terminal end. In some embodiments, the secretory signal is a synthetic sequence. In specific embodiments, the synthetic sequence is MLLK VVFA IGCI WQA (SEQ ID NO: 7). In yet further embodiments, the secretory signal is any signal that can induce secretion of the encoded protein, including without limitation an interleukin-2 secretory signal and a chymotrypsinogen secretory signal.

#### *Vargula* Luciferases of the Invention

In some aspects, the present invention provides a *Cypridina* Luciferase or *Vargula* luciferase (VLuc) from the marine ostracod *Vargula Hilgendorfi*, which is a secreted luciferase (emission max 395 nm or 462 nm depending on the substrate used).

In further aspects, the present invention provides a modified *Vargula* luciferase that shows increased signal and stability. In certain embodiments, the modified *Vargula* luciferase of the invention is human codon optimized to increase expression in mammalian systems. In further

embodiments, a modified *Vargula* luciferase of the invention includes a wildtype or a native human codon optimized luciferase with the last two amino acids have been mutated CQ to SN (S=serine, N=asparagine). In still further embodiments, the present invention provides a mammalian vector expressing modified human codon optimized *Vargula* luciferase expressing intracellular *Vargula* luciferase. This sequence is the same as the wildtype or native human codon *Vargula* luciferase with the last two amino acids mutated CQ to SN (S=serine, N=asparagine) and with a KDEL (endoplasmic reticulum retention) sequence added after the C-terminal asparagine residue.

#### Firefly Luciferases of the Invention

In some aspects, the present invention provides a red-emitting firefly luciferase (Red-Fluc) from the Italian firefly *Luciola Italica* (emission max 609 nm) and a green-emitting firefly luciferase (Green-Fluc) from the Italian firefly *Luciola Italica* (emission max 550 nm).

In further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases. Such human codon optimized red-emitting *L. Italica* luciferases show significantly increased activity over wildtype red-emitting *L. Italica* luciferases (see FIG. 8). In still further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases according to the sequence provided in FIG. 30 (SEQ ID NO: 3). In still further embodiments, the present invention provides human codon optimized sequences of red-emitting *L. Italica* luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 3. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 3.

In still further embodiments, the present invention provides secreted red-*Italica* luciferases. FIG. 10 shows a comparison of luciferase activity of a human codon optimized red-emitting *L. Italica* luciferases fused to a chymotrypsinogen secretory signal to a non-secreted form of the human codon optimized red-emitting *L. Italica* luciferase. As discussed above, a number of different secretory signals can be used to produce secreted forms of modified luciferases of the invention. However, for red firefly luciferase, not all secretory signals produce a secreted luciferase. For example, popular signal sequences such as the N terminal 16 amino acid sequence of *Gaussia* luciferase and the Interleukin 2 secretory sequence do not successfully produce a secreted form of red emitting firefly luciferase.

Fusing a chymotrypsinogen secretory signal to a human codon optimized red-emitting *L. Italica* luciferases did successfully produce a secreted form of this luciferase. In some embodiments, the present invention provides a red firefly luciferase (also referred to herein as "red-emitting luciferase" and "red-emitting *L. Italica* luciferase") that is modified to include a synthetic secretory signal. In certain embodiments, the modified red firefly luciferase is encoded by the polynucleotide has the sequence provided in FIG. 4 (SEQ ID NO: 2). In still further embodiments, the present invention provides a luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 2. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 2. The underlined portion of FIG. 4 is the secretory signal. FIG. 11 shows a comparison of luciferase activities in supernatants and lysates of HEK293 cells transfected with a secreted red *Italica* Luciferase of the invention.



In further embodiments, the present invention provides human codon optimized sequences of green-emitting *L. Italica* luciferases. Such human codon optimized green-emitting *L. Italica* luciferases show significantly increased activity over a previously described thermostable mutant of green-emitting *L. Italica* luciferase (B. R. Branchini et al., Analytical Biochemistry, 361 (2): 253-262 (2007)—see FIG. 9). In still further embodiments, the present invention provides human codon optimized sequences of green-emitting *L. Italica* luciferases according to the sequence provided in FIG. 31 (SEQ ID NO: 4). In still further embodiments, the present invention provides human codon optimized sequences of luciferases encoded by polynucleotides with about 80%-99% sequence identity to SEQ ID NO: 4. In still further embodiments, the present invention provides luciferases that are encoded by polynucleotides with about 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% sequence identity to SEQ ID NO: 4.

#### *Renilla* Luciferases of the Invention

In some aspects, the present invention provides a modified efficiently secreted blue-emitting (emission max 480 nm) *Renilla* luciferase (B-Rluc), which more stable than the wild-type *renilla reniformis* luciferase, and a green emitting secreted *Renilla* luciferase (emission max 535 nm) modified to be secreted by fusing a synthetic secretory signal encoding gene sequence in frame with the gene encoding the green emitting modified of *Renilla* luciferase. Mammalian cells transfected with the secreted green *Renilla* luciferase mutant described here show approximately 35-fold higher luciferase activity compared to mammalian cells transfected with the native (human codon optimized) *Renilla* luciferase (see FIG. 7). Further the secreted green *Renilla* luciferase shows excellent stability of the bioluminescent signal (without compromising signal intensity) when assayed using the *Renilla* luciferase assay reagent described in this application (with the stabilizer included, see FIG. 7), thus making it an ideal reporter for High throughput screening applications.

In certain embodiments, the present invention provides a green-emitting *Renilla* luciferase plasmid sequence with the sequence pictured in FIG. 3 (SEQ ID NO: 1).

#### *Gaussia* Luciferases of the Invention

In some aspects, the present invention provides a *Gaussia* luciferase (emission max 482 nm) that is either native secreted (Gluc) or modified to be expressed intracellularly (Gluc-KDEL). Such *Gaussia* luciferases can be used in single, double and triple reporter assays as discussed in further detail herein in combination with any of the other luciferases discussed herein or known in the art.

#### Luciferase Assays of the Invention

In certain aspects, the present invention provides compositions that improve stability and signal for assays utilizing wildtype and/or modified luciferases of the present invention.

In some embodiments, sodium chloride is added to improve the stability of luciferase assays of the invention. In such embodiments, a concentration of sodium chloride is utilized that improves the stability of the bioluminescent signal without affecting intensity. In further embodiments, sodium chloride concentrations in the range of about 0.05 M to about 1 M are used to improve stability of luciferase assays of the invention. In still further embodiments, sodium chloride concentrations of about 0.05 to about 0.5, 0.1 to about 0.4, about 0.2 to about 0.3, and about 0.05 to about 0.2M are used in luciferase assays of the invention. In specific embodiments, sodium chloride is added to improve the stability of assays utilizing wildtype and/or modified *Vargula* luciferases.

In further embodiments, certain luciferase substrates are added to luciferase assays to improve the stability of the bioluminescent signal. In such embodiments, the substrate added as a stabilizer may be an additional substrate that is not the substrate upon which the luciferase itself acts. For example, in assays utilizing *Cypridina* luciferase, coelenterazine is added to the assay to stabilize the assay stability. Coelenterazine is an oxidizable luciferin that is easily prone to oxidation but is not a substrate for the *Cypridina* luciferase. As will be appreciated, any luciferase assay described herein can be further modified by adding substrates for other luciferases as a stabilizer.

In some embodiments, the concentration of luciferase substrate is adjusted to improve the magnitude and/or stability of the signal. In further embodiments, low (under 1  $\mu$ M) concentrations of substrate is used to improve luciferase signals. For example, for *Cypridina* luciferase assays, about 1 to about 25 nM Vargulin are used in assays of the invention. In further embodiments, about 1-100, 5-90, 10-80, 15-70, 20-60, 25-50, and 30-40 nM Vargulin are used in assays of the invention. In further exemplary embodiments, substrates for the luciferase assays described herein (including *Cypridina*, *Gaussia* and *L. Italica* luciferases) are added in concentrations of from about 1 nM to about 250  $\mu$ M. In still further embodiments, substrates are added in concentration of about 10 nM-200  $\mu$ M, 50 nM-150  $\mu$ M, 100 nM-100  $\mu$ M, 150 nM-50  $\mu$ M, 200 nM-25  $\mu$ M, 300 nM-10  $\mu$ M, 500 nM-1  $\mu$ M.

In some embodiments, *Gaussia* luciferases of the invention are used with optimized reagents to produce increased activity. Kinetics of the *Gaussia* luciferase assay using the GAR-1 reagent is shown in FIG. 14. Measurement of the luciferase activity in supernatants of cells (transfected with *Gaussia* luciferase) using GAR-1 reagent from Targeting systems showed increased activity from *Renilla* luciferase assays from another vendor. The data in FIG. 14 is presented as an average of triplicate determinations measured on a Turner TD2020 luminometer. GAR-1 reagent has been described in detail in US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to assay reagents for the *Gaussia* luciferase assay.

In certain embodiments, *Gaussia* luciferase assays of the invention utilize reagents stabilized with stabilizing agents. In one non-limiting example, the stabilizing agents include NP40 (Sigma) and/or coelenterazine. In certain embodiments, about 5 to about 200  $\mu$ M coelenterazine is used. In still further embodiments, about 10-150, 20-125, 30-100, 40-75, 50-60  $\mu$ M coelenterazine is used. In yet further embodiments, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100  $\mu$ M coelenterazine is used. Stability of *Gaussia* luciferase assays using the GAR-2 reagent are shown in FIG. 15. Using the GAR-2B version of the *Gaussia* luciferase assay reagent, the bioluminescent signal remains very stable (FIG. 15A) In the absence of the stabilizer, the signal intensity is a little higher initially but decays faster than in the presence of the stabilizer (FIG. 15B). Note that the data presented in FIG. 15A and B is an average of triplicate determinations measured on a Turner TD2020 luminometer. The GAR-2 and GAR-2B reagents are stabilized versions of the GAR-1 reagent discussed in US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to reagents for *Gaussia* luciferase assays. The GAR-2 reagent includes the composition GAR-1 with an additional 30  $\mu$ M coelenterazine. GAR-2B reagent includes the composition GAR-1 with and additional 75  $\mu$ M coelenterazine. Without being limited by theory, it is possible that the higher (approximately 3-fold) signal



intensity seen with the GAR-2B reagent is due to the higher concentration of coelenterazine. FIG. 12B shows the stability of the *Gaussia* luciferase with the GAR-2 reagent including a stabilizer.

In certain embodiments, stability of firefly luciferase assays is improved using FLAR-1 reagents (Targeting Systems). FIG. 16 shows the results from experiments using the FLAR-1 reagent from Targeting Systems. In the experiments shown in FIG. 16, the FLAR-1 reagent was added to the supernatant cell culture media.

#### Dual and Triple Luciferase Assays

In some aspects, the present invention provides dual luciferase assays based on spectral resolution of two or more different luciferases. As will be appreciated, these assays can include different wildtype luciferases, different modified luciferases, or a mixture of a wildtype and a modified luciferase. Such assays rely on differences in the emission spectra of the reporters used. In further embodiments, reagents are modified to allow for more efficient multiplexing. For example, when *Gaussia* luciferases are multiplexed with firefly luciferases, EDTA is omitted from the reaction mixture to allow efficient reporter activity.

FIG. 13A shows the emission spectra of a dual reporter assay utilizing a *Vargula* and Red *Italica* luciferase of the invention. The luciferases were expressed in samples of transfected cells. The luciferases used in the experiments pictured in FIG. 13A represent a modified red emitting firefly luciferase of the invention that is human codon optimized and intracellular (non-secreted) and a *Cypridina* luciferase of the invention that is from *Cypridina hilgendorfi* modified to be human codon optimized and secreted.

FIG. 13B shows the emission spectra of a triple reporter assay utilizing *Vargula*, Green *Renilla* and Red *Italica* luciferases. These emission spectra were in samples of transfected cell lysates. The *Vargula* and red-emitting firefly luciferases are those as described above for FIG. 13A and the Green *Renilla* luciferase is an improved secreted Green luciferase mutant as described in further detail herein.

All patents and other references cited in the specification are indicative of the level of skill of those skilled in the art to which the invention pertains, and are incorporated by reference in their entireties, including any tables and figures, to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to obtain the ends and advantages mentioned, as well as those inherent therein. The methods, variances, and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention

that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Also, unless indicated to the contrary, where various numerical values or value range endpoints are provided for embodiments, additional embodiments are described by taking any 2 different values as the endpoints of a range or by taking two different range endpoints from specified ranges as the endpoints of an additional range. Such ranges are also within the scope of the described invention. Further, specification of a numerical range including values greater than one includes specific description of each integer value within that range.

Thus, additional embodiments are within the scope of the invention and within the following claims.

## EXAMPLES

### Example 1

#### Transfection of Mammalian Cells with Modified Luciferases

HEK-293 cells were grown in DMEM/10% FBS (fetal bovine serum) and transfected with plasmids expressing either the human codon-optimized or non-human codon optimized forms of the red emitting and green emitting firefly luciferases (from *Luciola Italica*) under control of the CMV promoter. Transfections were performed using the Targefect F-2 reagent (Targeting Systems) using the manufacturers protocols. Forty eight hours post transfection, the cells were lysed using the cell lysis reagent (CLR-1) from Targeting Systems, Santee. 20  $\mu$ l aliquots of the cell lysate were mixed with 100  $\mu$ l of the FLAR-1 (firefly luciferase assay reagent from Targeting Systems).

### Example 2

#### *Cypridina* Luciferase Assays with Increased Stability

Compositions were developed for achieving optimal performance of *Cypridina* luciferase assay reagents. These assays had improved stability of the bioluminescent signal without affecting the overall activity of the enzyme.

Vargulin is generally unstable and easily oxidized, making long term storage of this substrate difficult. However, Vargulin stored in an acidic buffer (66 mM monobasic potassium phosphate, pH 6-6.5) and stored at  $-80^{\circ}$  C. was very stable and did not lose activity even when stored for several months. In contrast, Vargulin dissolved in a neutral to basic phosphate buffer (e.g. 200 mM dibasic potassium phosphate (pH 8)) is very unstable and begins to lose activity rapidly within a few hours at room temperature. *Cypridina* luciferase activity was



## 11

optimal when 200 mM dibasic potassium phosphate was used as the reaction buffer instead of 66 mM monobasic sodium phosphate. Hence 200 mM dibasic potassium phosphate was used as the reaction buffer. Concentrations of be 3-6 mM Vargulin were found to be effective, and these concentrations are much lower than what is generally used in such assays (see for example Wu et al (2007) *Biotechniques*, 42(3):290-292).

The *Cypridina* luciferase assay showed increased stability when sodium chloride was included in the reaction. For example, FIG. 1 shows the relative luciferase stability (RLS) between VLAR-1 (no sodium chloride) and VLAR-2 (VLAR-1+sodium chloride). Sodium chloride clearly stabilized the RLS. For the experiments in FIG. 1, 20  $\mu$ l of sample was added with 40  $\mu$ l of VLAR solution for the assay followed by 20  $\mu$ l of Vargulin substrate.

FIG. 5 shows a further titration experiment indicating that sodium chloride concentrations of around 0.5M provide increased stability over control reagents with no sodium. Further concentrations that are of use in stabilizing such assays include from about 25 mM to about 750 mM sodium chloride. For experiments in FIG. 5, 5  $\mu$ l of the indicated concentrations of sodium chloride solutions were added to 35  $\mu$ l of VLAR buffer (20 mM dibasic potassium phosphate, pH=8.0). The assay was carried out by mixing 20  $\mu$ l of sample with 40  $\mu$ l of VLAR buffer (with sodium chloride) and then adding 20  $\mu$ l of *Cypridina* luciferin.

Further stability of the bioluminescent signal as well as improvement in overall luciferase activity was observed when coelenterazine, another oxidizable luciferin easily prone to oxidation (but not a substrate for *Cypridina* luciferase) was included in the assay composition. A 15 minute pre-incubation was found to result in increased stability of the bioluminescent signal using sample volumes between 5 and 20  $\mu$ l (roughly 40% drop in 26 minutes using an assay volume of 20  $\mu$ l and 15% drop in 26 minutes using an assay volume of 5  $\mu$ l—see FIGS. 2A and 2B. A concentration of coelenterazine that worked well to stabilize the reagent was 15  $\mu$ M. Concentrations in the range of about 10  $\mu$ M to about 50  $\mu$ M can also be used. The inclusion of coelenterazine in the composition decreased the background of the assay by more than 10-fold (background reading dropped from 153.6 to 12.4) and also resulted in a 15% increase in the intensity of the bioluminescent signal. Controls in which buffers with identical composition (i.e., inclusion of coelenterazine but omission of *Cypridina* luciferin) showed no activity. Coelenterazine is not a substrate for *Cypridina* luciferase and can be used to safely reduce the background and increase stability when *Cypridina* luciferase is assayed alone or in combination with other luciferases (such as firefly luciferase) which do not use coelenterazine as a substrate. For the experiments shown in FIG. 2, 5 or 20  $\mu$ l of the sample (media supernatant) was mixed with 40  $\mu$ l of the VLAR buffer (200 mM dibasic potassium phosphate, 50 mM NaCl). The firefly and *Cypridina* luciferase assay reagents can be mixed into a single solution which can be used to efficiently measure both *Cypridina* luciferase and firefly luciferase activity by spectrally resolving the luciferases using appropriate filters. However, the DTT concentration in the firefly luciferase assay reagent can affect activity in such situations, because the activity of both luciferases is decreased due to interference of DTT (present in low concentration in the firefly assay reagent with the *Cypridina* luciferase assay (there is almost a 10-fold drop in *Cypridina* luciferase activity). However, since the signal intensity of the *Cypridina* luciferase assay is very robust, the signal is still acceptable and improvement in *Cypridina* luciferase activity is observed if the DTT concentration in the

## 12

firefly luciferase assay reagent is dropped to 2.5 mM (a 3 fold drop in activity of *Cypridina* luciferase is still observed). Single solution based dual assays in which *Cypridina* luciferase is multiplexed with Green emitting *Renilla* luciferase work very well without loss of activity of either *Cypridina* or *renilla* luciferase when the two solutions are mixed.

## Example 3

### *Renilla* Luciferase Assays Utilizing Modified *Renilla* Luciferases and Stabilizing Reagents

The secreted modified green *Renilla* luciferase of the present invention showed significantly greater activity over wildtype *Renilla* luciferase—see FIG. 7. For the experiments pictured in FIG. 7, HEK 293 cells were transfected with expression vectors expressing either native *Renilla* luciferase or the secreted Green *Renilla* luciferase mutant. Cells were lysed 48 hrs post transfection and assayed for luciferase activity.

Assays with and without stability assay reagents for green *Renilla* luciferase were investigated. FIG. 6 shows that assays conducted with stabilizer showed greater stability than those without. The composition of the *Renilla* luciferase assay reagent (no stabilizer) was: 30  $\mu$ M coelenterazine, 0.4 $\times$ PBS (Ca, Mg free), 0.027% NP40. The composition of the *Renilla* luciferase assay reagent (with stabilizer) was: 30  $\mu$ M coelenterazine, 0.4 $\times$ PBS (Ca, Mg free), 0.227% NP40. Stabilizer is 2% NP40 (a non-ionic detergent).

## Example 4

### Kinetics of Different Luciferases

Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the luciferase assay reagents supplied with the LiveResponse assay kit. These data are shown in FIG. 12A: Red *Luciola* (firefly), luciferase, FIG. 12B *Gaussia Princeps* luciferase (this is FIG. 15C), FIG. 12C: *Cypridina* luciferase, and FIG. 12D: Green *Renilla* luciferase. Data represents mean of triplicate determinations.

## Example 5

### Comparison of Expression Vectors Expressing Modified *Vargula* Luciferases

Transfection protocols were as follows: HEK-293 cells were grown in DMEM/10% FBS (fetal bovine serum) and transfected with plasmids expressing either the human codon-optimized to non-human codon optimized forms of native VLuc, HC-VLuc, sequence 1) or modified HC-VLucs under control of the CMV promoter. Transfections were performed using the Targefect F-2 reagent (Targeting Systems) using the manufacturers protocols.

The stability of the bioluminescent signal of *Cypridina* Luciferase assessed using supernatants of HEK293 cells transiently transfected with the pCMV VLuc expression vector is shown in FIG. 17.

In FIG. 19, the stability of the bioluminescent signal of *Cypridina* Luciferase was assessed using supernatants from HEK 293 cells transiently transfected with the pCMV-VLuc expression vector. Samples were assayed using the VLAR-2



## 13

(VLAR-1 reagent from Targeting Systems with sodium chloride) of the *Cypridina* luciferase assay reagent.

Human codon optimization of the gene sequence encoding the VLuc led to a 5-fold improvement of luciferase expression in HEK-293 transfected with expression vectors containing the human codon optimized versions of the *vargula* luciferase genes compared to the native sequences (i.e. Non-human codon optimized sequences). Addition of the KDEL sequence at the C-terminal end results in intracellular expression of VLuc.

## Example 6

Construction of Blue-Emitting (Blue Shifted) and Green Emitting Mutants of Secreted *Renilla* Luciferase for Use as Secreted Reporters in Single or Multiplexed Luciferase Assays

A synthetic signal peptide was deduced by rational design: MLLK VVFA IGCI VVQA (SEQ ID NO: 7). The sequence of this signal peptide was based on rational design using signal sequences from the secretory signals known in the art, including those available at: <http://www.unitargeting.com/Resources/Trends07.pdf>

Blue-Shifted Secreted *Renilla* Luciferase Mutants

Secreted mutants were constructed containing signal peptide fused to amino terminal region of the human codon optimized *renilla reniformis* luciferase with the following additional mutations which enable i) efficient refolding after secretion to obtain an active form of the enzyme (Loma Linda paper, cysteine 124 was mutated to alanine) and additional mutations to cause a shift in the emission max of *Renilla* luciferase. MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; N53Q; V146M. Emission maxima=475 nm

Secreted BLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; N53Q; V146M and the following eight additional mutations A55T, S130A, K136R, A143M, M185V, M253L, S287L. The 8 additional mutations increase intensity of the bioluminescent signal (Emission Maxima 475 nm)

Red Shifted *Renilla* Luciferase Mutants:

Secreted RLuc Sequence 1: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A, D162E

Secreted RLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L F262W. Emission Maxima 535 nm

Secreted RLuc Sequence 3: MLLK VVFA IGCI VVQA-HCRLuc with following mutations C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L. Emission Maxima 535 nm

## Example 7

Tests for Developing Assays for *Vargula* Luciferase

In some embodiments, different buffer solutions are used to improve assays utilizing wildtype and/or modified luciferases of the invention. In certain embodiments, a 1:1 mixture of 0.1 M Tris HCl and 75 mM sodium phosphate is used as the assay buffer.

Several different parameters were tested to develop an assay for *vargula* luciferase:

Effects of using either an acidic buffer (e.g., potassium phosphate pH 5-6.8), Tris HCl pH 7.4, Tris phosphate buffer pH (8-8.5) as well as varying assay volumes were tested. In

## 14

general the use of acidic conditions significantly reduced the intensity of the bioluminescent signal (typically 5-10 fold) while increasing the stability somewhat. Using Tris HCl pH 7.4, the activity as the assay buffer resulted in 5-10 fold brighter bioluminescence but the luminescent signal was highly unstable.

Use of a buffer mixture (1:1) of 50 mM Tris HCl, pH 7.4 and 100 mM dibasic sodium phosphate resulted in improved stability of the bioluminescent signal without compromising the intensity of the bioluminescent signal. An interesting finding was that inclusion of 0.2 M NaCl further increased stability of the bioluminescent signal. Lastly the amounts of Vargulin needed for optimal activity using this buffered condition are very low (1-10 nM range) making the assay extremely useful and economical.

Increasing the concentration of Vargulin further did not increase stability of the assay further.

Stock Vargulin substrate solutions stored in an acidic condition pH (5.5-6) were relatively stable over several months when stored at -80° C.

Other parameters tested: Other stabilizers such as DTT (dithiothreitol), detergents like NP-40 or EDTA were unable to increase the intensity of the luminescent signal or improve stability of the assay. EDTA decreased the VLuc activity by at least 5-fold.

Thus one aspect of the invention concerns the following composition and variations thereof: 20 µl of cell supernatant assays with 50 µl of Tris/phosphate buffer, pH 8, 0.2 M NaCl, 10 µl of 5-100 nM vargulin in 66 mM potassium phosphate (monobasic). In certain assays, the effective concentration of vargulin in the assay mix is as low as 20 nM which is approximately 50-fold lower than that reported in the literature (see for example Wu et al (2007) Biotechniques, 42(3):290-292)

Comparison of luciferase activity in cells transfected with *vargula* luciferase with luciferase activity in cells transfected with firefly luciferases from *Photinus pyralis* or *Luciola Italica* showed that *vargula* luciferase was a much more sensitive reporter (10-20 fold improvement in bioluminescent signal compared to firefly luciferase, assay done in HEK-293 cells, all expression vectors were expressed luciferase under control of the CMV promoter). An exemplary assay protocol included: 20 µl aliquots of Cell supernatants (media with 5% serum) were mixed with 100 µl of assay dilution buffer (50 µl of 50 mM TrisHCl, 100 mM dibasic sodium phosphate, pH 8) and 10 µl of vargulin in sodium phosphate buffer pH 6 (final concentration of vargulin in reaction mix 10-25 nM). The sample was mixed well and bioluminescent activity was recorded in a Turner TD2020 luminometer integrated over a 20 sec time interval.

## Example 8

Activity in Cell Supernatant and Cell Lysates of Cell Transfected with Either a Plasmid Vector Expressing Secreted *Vargula* Luciferase or an Intracellular Form of *Vargula* Luciferase

In cells transfected with the secreted form of modified *vargula* luciferase, 80% of the activity was secreted into the cell supernatant and only 20% is cell-associated.

FIG. 18 shows intracellular and secreted *Cypridina* luciferase activity. Luciferase activity in cell supernatants and cell lysates of cells transfected with a plasmid vector expressing secreted *vargula* luciferase. As shown in FIG. 18 cells transfected with the secreted form of modified *vargula* luciferase, 80% of the activity is secreted into the cell supernatant and only 20% is cell-associated.



## 15

In cells transfected with *vargula* luciferase modified at the C-terminal end with a KDEL sequence, approximately 95% of the activity was intracellular and 5% is secreted.

## Example 8

Development of a Dual Reporter System Based on Blue and Red Shifted Mutants of Secreted *Renilla* Luciferase

Secreted mutants: Secreted mutants were constructed containing signal peptide fused to amino terminal region of the human codon optimized *renilla reniformis* luciferase with the following additional mutations which enable i) efficient refolding after secretion to obtain an active form of the enzyme (Cysteine 124 was mutated to alanine) and additional mutations to cause a shift in the emission max of *renilla* luciferase: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; N53Q; V146M. Emission maxima=475 nm

Secreted RLuc Sequence 2: MLLK VVFA IGCI VVQA-HCRLuc with following mutations. C124A; N53Q; V146M and the following eight additional mutations A55T, S130A, K136R, A143M, M185V, M253L, S287L. The 8 additional mutations increase intensity of the bioluminescent signal. Emission Maxima 475 nm

RED SHIFTED *RENILLA* LUCIFERASE MUTANTS: Secreted RLuc Sequence 1: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A, D162E

Secreted RLuc Sequence 3: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; and the following eight additional mutations AI23S/D154M/E155G/D162E/I163L/V185L F262W. Emission Maxima 535 nm

Secreted RLuc Sequence 4: MLLK VVFA IGCI VVQA-HCRLuc with following mutations: C124A; and the following eight additional mutations. A123S/D154M/E155G/D162E/I163L/V185L. Emission Maxima 535 nm

A single solution dual luciferase assay based on secreted *renilla* luciferase blue emitting (emission max at 475 nm) and green emitting mutants (emission max at 535 nm).

The mutations in the above sequences lead to the efficient expression of secreted *renilla* luciferase in the transfected cells. The two luciferases can therefore be used in combination as a dual reporter system and the luciferase activity of each luciferase in the transfected cells can be resolved by using appropriate filters. The reagent compositions for *renilla* luciferase assay reagents are described Walia, US Pat Appl Publ 2008074485, entitled Enhancing a Luminescent Signal, which is incorporated herein by reference in its entirety and in particular for all teachings related to *Renilla* luciferase assay reagents.

## Example 9

Development of a Triple Reporter System Based on Red and Green Emitting Firefly Luciferases and *Gaussia* Luciferase/*Renilla* Luciferase

Composition of the *Gaussia* luciferase assay reagent (GAR-1) has been described in detail in a US Pat Appl Publ 2008074485, which is hereby incorporated by reference in its entirety and in particular for all teachings related to assay reagents for the *Gaussia* luciferase assay. An assay reagent useful for simultaneous measurement of all three reporters in a single solution was designed by omitting EDTA from the composition of the *Gaussia* luciferase assay reagent and then including all the ingredients necessary for assay of firefly

## 16

luciferase in a single composition. The rationale behind this is that the EDTA interferes with the firefly luciferase assay (magnesium is an important co-factor for firefly luciferase and EDTA chelates magnesium). The ingredients required for Firefly luciferase assay included in the assay composition were as follows—ATP, DTT. Firefly luciferin, magnesium sulfate, magnesium bromide (helps increase brightness of luminescent signal) and phosphate buffer.

The composition of the single solution for a triple reporter assay for measuring *Gaussia* luciferase or *Renilla* luciferase in combination with red and green emitting firefly luciferase is as follows:

0.1×PBS. 5.4 ml of 5% NP40 diluted to 1000 ml and add the following:

To 800 ml of the above solution add the following:

Tricine 3.227 g (20 mM)

1M Magnesium sulfate 0.7H<sub>2</sub>O 2.51 ml (2.67 mM)

Magnesium bromide 0.6 H<sub>2</sub> (1.07 mM)—add 2.14 ml of 500 mM stock solution

25 mM OTT (3.86 g)

530 μM ATP (2.72 g)

CoA (0.18 g)—optional

Adjust with sodium phosphate to pH 7.8

Add 940 μM D-Luciferin (free acid)—253.81 mg

CDTA-0.8289 g

940 μM D-luciferin (free acid)—253.81 mg

CDTA-0.8289 g

0.8M Tris (0.02 M EDTA)—43.53 ml

Add GAR reagent without EDTA to a total volume of 1 liter

Dilute 100× coelenterazine substrate with the above solution to 1× just before use. Use normal 3 mg/5 ml absolute alcohol acidified with 30 μl of 2N HCl)

NOTE: This assay reagent does not contain enough cell lysis reagents. Hence cells have to be first lysed using 1× Cell Lysis Buffer (compatible with use of all luciferases (prepared from 5× stock solution described below:

Dilute the 5× Cell lysis buffer described below with water to 1× concentration and add to washed cells and shake at 400 rpm for 20 mins to lyse cells.

Composition of 5× Cell Lysis Buffer:

For 1 liter of Buffer

5 ml NP 40 (undiluted)

25 ml Tris HCl pH 8

1.45 g NaCl

50 ml glycerol

## Example 10

Development of a Single Solution Triple Luciferase Reporter Assay Based on Red and Green Emitting Firefly Luciferases and *Vargula* Luciferase

A *vargula* luciferase-based triple reporter system was prepared by first preparing the *vargula* luciferase assay reagent (VLAR-1) and mixing it in a 1:1 ratio with the firefly luciferase assay reagent (FLAR-T) to give the triple assay reagent TVLAR-1.

Assay protocol: To 20 μl of cell lysate add 100 μl of the TVLAR-1 reagent and read in the Victor luminometer (Perkin Elmer) or Varian (Promega) using appropriate filters.

Preparation of VLAR-1 Reagent:

Composition of the *Vargula* Luciferase Assay reagent is described below

500 ML OF 0.1 M TRIS HCL PH 8

500 ML of dibasic sodium phosphate 200 mM

200 ml of 5 nM Vargulin in 66 mM potassium phosphate pH 5.5



## 17

pH of final solution is 8-8.5

Composition of the FLAR-T Reagent

SOLUTION A: 0.1×PBS. 5.4 ml of 5% NP40 diluted to 1000 ml and add the following:

To 800 ml of the above solution add the following:

Tricine 3.227 g (20 mM)

1M Magnesium sulfate. 7H<sub>2</sub>O 2.51 ml (2.67 mM)

Magnesium bromide (0.6 H<sub>2</sub>) (1.07 mM)—add 2.14 ml of 500 mM stock solution

5 mM DTT (in some embodiments, any range between 5 mM and 30 mM can be used, including 5, 10, 15, 20, 25, 26, 27, 28, 29, and 30 mM)

530 μM ATP (2.72 g)

CoA (0.18 g)—optionally omitted

Adjust with sodium phosphate to pH 7.8

Add 940 μM D-Luciferin (free acid)—253.81 mg

CDTA-0.8289 g

940 μM D-luciferin (free acid)—253.81 mg

CDTA-0.8289 g

941 μM D-luciferin (free acid)—253.81 mg

CDTA-0.8289 g

0.8M Tris (0.02 M EDTA)—43.53 ml

ADD SOLUTION A to a total volume of 1 liter

NOTE: This assay reagent does not contain enough cell lysis reagents for effective lysis. Hence cells should first be lysed, e.g., using 1× Cell Lys is Buffer (compatible with use of all luciferases (prepared from 1× stock solution described below: Dilute the 5× Cell lysis buffer described below with water to 1× concentration and add to washed cells and shake at 400 rpm for 20 mins to lyse cells.

Composition of 5× cell lysis buffer:

For 1 liter of Buffer

5 ml NP 40 (undiluted)

25 ml Tris HCl pH 8

1.45 g NaCl

50 ml glycerol

Composition of Firefly luciferase assay reagent (for use of firefly luciferase as a single reporter gene).

20 mM tricine (179.2 3.55 g)

MgCo<sub>3</sub> 1.07 mM 0.55 g

Magnesium sulfate 2.7 mM (277 ml)

0.1 mM EDTA

20 mM DTT (4.25 g)

530 μM ATP (3 g)

CoA (0.198 g)

Add disodium phosphate 25 g to pH 7.8

Add 793 ml water before pH

470 μM D Luciferin free acid 279.2 mg

5×CCLR 307 ml

Composition of 5×CCLR:

0.8 M Tris 0.02 M EDTA pH 8-156 ml

Glycerol 500 ml

Triton X100 50 ml

CDTA-7.5 m moles (2.7 g)

DTT 10 mM 1.542 g total vol 1 liter.

## Example 11

Development of a Single Solution Triple Luciferase Reporter Assay Based on Red and Green Emitting Firefly Luciferases and *Vargula* Luciferase

Addition of stabilizer does not significantly affect (i.e., there is very little decrease in signal intensity) intensity of bioluminescent signal of *Renilla* luciferase in supernatants and lysates. FIG. 20 (top panel) shows a *Renilla* assay performed with 10 μl of *Renilla* Lysate and 20 μl of *Renilla*

## 18

Supernatant. Assay went as follows: 20 or 10 μl of sample (Supernatant or Lysate), 50 μl of RLAR-1 reagent (Targeting Systems). FIG. 20 (bottom panel) shows *Renilla* Assay was performed using the same volumes of lysate and supernatant as in the experiments in the top panel. Assay protocol was as follows: 10 or 20 μl of lysate or supernatant depending on the assay, 50 μl of the RLAR-1 reagent and an additional 8 μl of RLAR stabilizer for an increased stability profile for a time course reading. The stabilizer lowered the initial RLU reading (decreased from approximately 9000 to approximately 7000 rlu) but showed a much higher level of stability when observed over 30 minutes to 1 hour (FIG. 12C). The RLAR-1 reagent is useful for high throughput screening (HTS) applications in which a large number of samples need to be assayed. In the absence of the stabilizer, the signal intensity decays faster than in the presence of stabilizer (FIG. 21). Note: Data presented is average of triplicate determinations measured on a Turner TD2020 luminometer. In FIG. 21, a time course was taken using the standard protocol of 10 μl lysate, 50 μl of RLAR reagent without stabilizer indicating drop in *Renilla* luciferase activity.

FIG. 22 shows the stability of the bioluminescent signal of *Cypridina* luciferase and firefly luciferase using the DLAR-3 reagent. This reagent is useful for HTS applications involving both *Cypridina* luciferase and the red-emitting *Luciola* luciferase. Note: Data presented is average of triplicate determinations measured on a Turner TD2020 luminometer. The DLAR-3 reagent (Targeting Systems) is a dual assay reagent based on secreted *Cypridina* luciferase and a secreted or intracellular red-emitting firefly luciferase.

FIG. 28 shows emission spectra of *Cypridina* and Firefly luciferases in samples of transfected cells (lysates or supernatants). The emission spectra were recorded on a Fluorolog-3 spectrofluorometer (Horiba Scientific, Japan) using a liquid nitrogen cooled CCD. The luciferases were assayed by mixing 200 ul of the sample with the appropriate luciferase assay reagent to obtain spectral profiles. Emission max of *Cypridina* Luciferase is 463 nm; Red *Italica* 617 nm.

## Example 12

Double and Triple Luciferase Reporter Assays Based on *Renilla* Luciferase, Firefly Luciferase and *Vargula* Luciferase

Kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the DLAR-5 system are shown in FIG. 23. Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the DLAR-5 luciferase assay reagents. The decay of the *renilla* luciferase signal shown in Panel B above can be greatly minimized (ie the bioluminescent signal can be rendered much more stable by addition of a *Renilla* luciferase stabilizer to the DLAR-5 buffer.

FIG. 24 shows Emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activities of *Cypridina*, Green *Renilla* luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of *Vargula* luciferase is 463 nm; Green *Renilla* luciferase is 527 nm. Note that the data presented in this application is performed with the green-emitting mutant that emits at 527 to 530 nm (this is the variation in emission maxima seen and the luciferase is different in sequence, properties and emission maximum from the 535 nm emitting intracellular green emitting *Renilla* luciferase mutant described in US Patent Publication No.



20090136998, which is hereby incorporated by reference in its entirety and in particular for all teachings related to Green *Renilla* luciferase.

FIG. 25 shows kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the triple reporter system. Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were measured using the TLAR luciferase assay reagents (Targeting Systems).

FIG. 26 shows emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activi-

ties of *Cypridina*, *Renilla* and Red *Luciola Italia* luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of *Vargula* luciferase is 463 nm; Green *Renilla* luciferase is 527 nm and Red *Luciola Italia* luciferase is 617 nm.

The present invention also provides a single solution-based triple luciferase reporter assay involving *Cypridina* luciferase multiplexed with Green-emitting *Renilla* luciferase and Red-emitting Firefly luciferase. This assay is compatible with high throughput applications. This assay is also optionally in a format where the three luciferases can be assayed separately using three different assay reagents

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 7

<210> SEQ ID NO 1

<211> LENGTH: 6173

<212> TYPE: DNA

<213> ORGANISM: *Renilla reniformis*

<400> SEQUENCE: 1

```

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg      60
ccgcatagtt aagccagtat ctgctccctg cttgtgtggt ggaggteget gagtagtgcg      120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc      180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt      240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata      300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc      360
cccgccatt gacgtcaata atgacgatg ttcccatagt aacgccaata gggactttcc      420
attgacgtca atgggtggac tatttacggg aaactgcccc cttggcagta catcaagtgt      480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcttggcatt      540
atgcccgata catgacctta tgggactttc ctacttggca gtacatctac gtattagtca      600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggctgga tagcggtttg      660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc      720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc ccattgacg caaatgggcg      780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca      840
ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gcttgggtacc      900
gagctcggat ccatgttgtt gaaagttgtg tttgctattg gatgtatcgt agtgcaggct      960
atggcctcaa aagtgtacga tccggagcag cggaagagga tgatcacggg gcccacatgg     1020
tgggcacgat gcaagcagat gaatgtgttg gacagtttca ttaactacta cgacagcgag     1080
aaacacgcgg agaacgcagt gatattcctg cacggcaatg caaccagtag ctatctgtgg     1140
agacacgtgg tgctcatat tgagccggtc gctagatgca ttattcccga tcttattgga     1200
atggggaaat ccggaagag tggaaatgga tcatataggc tcctcgatca ttataaatat     1260
ctgactgctt ggtttgaatt gctcaatctg cccaagaaaa tcatctttgt aggacatgat     1320
tggggctccg cccttgcttt tcattatgcc tatgaacacc aggatcggat caaggctatt     1380
gttcacatgg agagcgtggt ggatgtgatt gaatcatgga tggggtggcc ggatatagaa     1440
gaagagctgg cgctgattaa atctgaggag ggcgagaaga tggactcga aaataacttc     1500
tttgtcgaga cggactgcc cagtaagatc atgcgcaaac tggagcctga agagtttgcg     1560
gcttacctgg aaccettcaa ggagaagga gaggtgagga gaccgacct gtcattggcct     1620

```

-continued

---

cgggaaattc	cgctggtcaa	aggaggggaag	ccagacgtcg	tcgccattgt	ccggaattac	1680
aacgcttacc	tccgcgctag	tgacgacctg	cctaaactct	tcategaatc	agatcctggt	1740
ttcttttagta	acgccatcgt	cgagggcgcc	aagaagtttc	caaacaccga	atttgttaaa	1800
gtcaaaggac	ttcacttctc	ccaggaggat	gcgcccgatg	aatgggaaa	gtatatcaaa	1860
tccttcgtgg	agaggtctt	gaagaatgag	cagaggtcca	tctagtctag	aaataattct	1920
tactgtcatg	ccaagtaaga	tgcttttctg	tgctgcaata	gcaggcatgc	tggggatgcg	1980
gtgggctcta	tggcttctga	ggcggaaaga	accagctggg	gctctagggg	gtatccccac	2040
gcgccctgta	gcggcgctt	aagcgcggcg	ggtgtggtgg	ttacgcgcag	cgtgaccgct	2100
acacttgcca	gcgccctagc	gcccgcctct	ttcgctttct	tccttctctt	tctcgccacg	2160
ttcgccggct	ttccccgtca	agctctaaat	cggggcatcc	ctttagggtt	ccgatttagt	2220
gctttacggc	acctcgacct	caaaaaactt	gattagggtg	atggttcacg	tagtggggcca	2280
tcgccctgat	agacggtttt	tcgccctttg	acgttgaggt	ccacgttctt	taatagtgga	2340
ctcttgttcc	aaactggaac	aacctcaac	cctatctcgg	tctattcttt	tgatttataa	2400
gggattttgg	ggatttcggc	ctattgggta	aaaaatgagc	tgatttaaca	aaaatttaac	2460
gcgaattaat	tctgtggaat	gtgtgtcagt	taggggtggt	aaagtcccca	ggctccccag	2520
gcaggcagaa	gtatgcaaag	catgcatctc	aattagtcag	caaccagggt	tggaaagtcc	2580
ccaggctccc	cagcaggcag	aagtatgcaa	agcatgcata	tcaattagtc	agcaaccata	2640
gtcccgcgcc	taactccgcc	catcccgcgc	ctaactccgc	ccagttccgc	ccattctccg	2700
ccccatggct	gactaatttt	ttttatttat	gcagaggccg	aggccgcctc	tgectctgag	2760
ctattccaga	agtagtgagg	aggctttttt	ggaggcctag	gcttttgcaa	aaagctcccc	2820
ggagcttgta	tatccatttt	cggatctgat	caagagacag	gatgaggatc	gtttcgcag	2880
attgaacaag	atggattgca	cgcaggttct	ccggccgctt	gggtggagag	gctattcggc	2940
tatgactggg	cacaacagac	aatcggctgc	tctgatgccg	ccgtgttccg	gctgtcagcg	3000
caggggcgcc	cggttctttt	tgtcaagacc	gacctgtccg	gtgccctgaa	tgaactgcag	3060
gacgaggcag	cgcggctatc	gtggctggcc	acgacgggcg	ttccttgccg	agctgtgctc	3120
gacgttgca	ctgaagcggg	aaggactggg	ctgctattgg	gcgaagtgcc	ggggcaggat	3180
ctcctgtcat	ctcaccttgc	tctgcccag	aaagtatcca	tcatggctga	tgcaatgcgg	3240
cggctgcata	cgcttgatcc	ggctacctgc	ccattcgacc	accaagcgaa	acatcgcatc	3300
gagcagacac	gtactcggat	ggaagccggg	cttgcgatac	aggatgatct	ggacgaagag	3360
catcaggggc	tcgcgccagc	cgaactgttc	gccaggctca	aggcgcgcag	gcccgcggc	3420
gaggatctcg	tcgtgacca	tggcgatgcc	tgettgcga	atatcatggt	ggaaaatggc	3480
cgttttctg	gattcatcga	ctgtggccgg	ctgggtgtgg	cggaccgcta	tcaggacata	3540
gcgttggtta	cccgtgatat	tgtgaagag	cttgccggcg	aatgggctga	ccgcttctc	3600
gtgctttacg	gtatcgccgc	tcccgattcg	cagcgcacag	ccttctatcg	ccttcttgac	3660
gagttcttct	gagcgggact	ctggggttcg	aatgaccga	ccaagcgacg	cccaacctgc	3720
catcacgaga	tttcgattcc	accgcccctt	tctatgaaag	gttgggcttc	ggaatcgttt	3780
tccgggacgc	cggctggatg	atcctccagc	gcggggatct	catgctggag	ttcttcgccc	3840
accccaactt	gtttattgca	gcttataatg	gttacaataa	aagcaatagc	atcacaattt	3900
tcacaaataa	agcatttttt	tactgcatt	ctagttgtgg	tttgtccaaa	ctcatcaatg	3960



-continued

---

tatcttatca	tgtctgtata	cogtcgacct	ctagctagag	cttggcgtaa	tcatgggtcat	4020
agctgtttcc	tgtgtgaaat	tgttatccgc	tcacaattcc	acacaacata	cgagccggaa	4080
gcataaagtg	taaagcctgg	ggtgcctaata	gagtgagcta	actcacatta	attgcggttg	4140
gctcactgcc	cgctttccag	tcgggaaacc	tgtcgtgcca	gctgcattaa	tgaatcggcc	4200
aacgcgcggg	gagaggcgg	ttgcgtattg	ggcgctcttc	cgcttctctg	ctcactgact	4260
cgctgcgctc	ggcgttcgg	ctgcggcgag	cggtatcagc	tcactcaaag	gcggtaatac	4320
ggttatccac	agaatcaggg	gataacgcag	gaaagaacat	gtgagcaaaa	ggccagcaaa	4380
aggccaggaa	ccgtaaaaag	gccgcggttc	tggcggtttt	ccataggctc	cgccccctg	4440
acgagcatca	caaaaatcga	cgctcaagtc	agaggtggcg	aaacccgaca	ggactataaa	4500
gataccaggc	gtttcccct	ggaagctccc	tcgtgcgctc	tcctgttccg	accctgccgc	4560
ttaccggata	cctgtccgcc	tttctccctt	cggaagcgt	ggcgctttct	caatgctcac	4620
gctgtaggta	tctcagttcg	gtgtaggtcg	ttcgctccaa	gctgggctgt	gtgcacgaac	4680
ccccggttca	gcccgaccgc	tgcgccttat	ccgtaacta	tcgtcttgag	tccaacccgg	4740
taagacacga	cttatcgcca	ctggcagcag	ccactggtaa	caggattagc	agagcgaggt	4800
atgtaggcgg	tgctacagag	ttcttgaagt	ggtggcctaa	ctacggctac	actagaagga	4860
cagtatttgg	tatctgcgct	ctgctgaagc	cagttacctt	cggaaaaaga	gttggtagct	4920
cttgatccgg	caaaaaacc	accgctggta	gcggtggttt	ttttgtttgc	aagcagcaga	4980
ttacgcgcag	aaaaaaagga	tctcaagaag	atcctttgat	cttttctacg	gggtctgacg	5040
ctcagtggaa	cgaaaactca	cgttaagggg	ttttggtcat	gagattatca	aaaaggatct	5100
tcacctagat	ccttttaaat	taaaaatgaa	gttttaaatc	aatctaaagt	atatatgagt	5160
aaacttggtc	tgacagttac	caatgcttaa	tcagtgaggc	acctatctca	gcgatctgtc	5220
tatttcggtc	atccatagtt	gcctgactcc	ccgtcgtgta	gataactacg	atacgggagg	5280
gcttaccatc	tggccccagt	gctgcaatga	taccgcgaga	cccacgctca	ccggctccag	5340
atztatcagc	aataaaccag	ccagccggaa	gggcccagcg	cagaagtgg	cctgcaactt	5400
tatccgcctc	catccagtct	attaattggt	gccgggaagc	tagagtaagt	agttcgccag	5460
ttaatagttt	gcgcaacggt	gttgccattg	ctacaggcat	cgtgggtgca	cgctcgtcgt	5520
ttggtatggc	ttcattcagc	tccggttccc	aacgatcaag	gcgagttaca	tgatccccca	5580
tgttggtgca	aaaagcgggt	agctccttcg	gtcctccgat	cgttgtcaga	agtaagttgg	5640
ccgcagtggt	atcactcatg	gttatggcag	cactgcataa	ttctcttact	gtcatgccat	5700
ccgtaagatg	cttttctgtg	actggtgagt	actcaaccaa	gtcattctga	gaatagtgta	5760
tgcggcgacc	gagttgctct	tgcccggcgt	caatacggga	taataccgcg	ccacatagca	5820
gaactttaa	agtgtcatc	attggaaaac	gttcttcggg	gcgaaaactc	tcaaggatct	5880
taccgctggt	gagatccagt	tcgatgtaac	ccactcgtgc	acccaactga	tcttcagcat	5940
cttttacttt	caccagcgtt	tctgggtgag	caaaaaacgg	aaggcaaaat	gccgcaaaaa	6000
aggaataaag	ggcgacacgg	aatggtgaa	tactcatact	cttccttttt	caatattatt	6060
gaagcattta	tcagggttat	tgtctcatga	gcggatacat	atgtgaatgt	atthagaaaa	6120
ataaacaat	aggggttccg	cgcacatttc	cccgaagaat	gccacctgac	gtc	6173

&lt;210&gt; SEQ ID NO 2

&lt;211&gt; LENGTH: 6839

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

-continued

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Modified red firefly luciferase with secretory signal

&lt;400&gt; SEQUENCE: 2

gacggatcgg gagatctccc gatcccctat ggtcgcactct cagtacaatc tgctctgatg 60  
ccgcatagtt aagccagtat ctgctccctg cttgtgtggt ggaggtcgct gagtagtgcg 120  
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180  
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240  
gattattgac tagttattaa tagtaatcaa ttacggggtc attagtcat agcccatata 300  
tggagtccg cgttacataa cttacggtaa atggcccgc tggctgaccg cccaacgacc 360  
cccgccatt gacgtcaata atgacgatg ttcccatagt aacgccaata gggactttcc 420  
attgacgtca atgggtggac tatttacggg aaactgcccc cttggcagta catcaagtgt 480  
atcatatgcc aagtagccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540  
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600  
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660  
actcacgggg atttccaagt ctccaccca ttgacgtcaa tgggagtttg ttttggcacc 720  
aaaatcaacg ggactttcca aaatgctgta acaactccgc ccattgacg caaatgggcg 780  
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca 840  
ctgcttactg gcttatcgaa attaatacga ctactatag ggagaccaa gcttgggtacc 900  
gagctcggat ccatggcctt cctgtggctg ctgtcctgct gggccctgct gggcaccacc 960  
ttcggctacc cgatcgagga gggctctgcc ggcaccaat tgcacaagta catgcaaaa 1020  
tacgccaagc tcggcgccat cgccttcagt aacgcccga caggcgtcga catcagctac 1080  
cagcagtact tcgacatcac gtgcagactc gccgaggcta tgaagaacta cggcatgaag 1140  
ccagaaggac acatcgtctt ctgtagcgag aactgcgaag agttcttcat tctgttctg 1200  
gctggtcttt acatcgagat tacagtcgcg ccaactaacg aaatttatac acttagagag 1260  
ctgaaccaca gtctggggat agcccaacct actatcgat tctctagcag gaagggcctg 1320  
cccaaagtgc ttgaggtgca gaagaccgtg acttgcacaa aaaccattgt catcctggac 1380  
agtaagggtca acttcggcgg ttatgactgc gtagagacct tcattaagaa acacgtcgag 1440  
ctgggctttc ctgccacctc atttgtgccc atcgacgtca aagaccgaa gcaccacatt 1500  
gctctgctta tgaactctc cggttccaca gggctgcccc aaggagtaga gatcactcac 1560  
gaggccctgg tcacgagatt ctctcacgct aaggacccta tatacggcaa tcaggtggcc 1620  
ccaggtaccg ctatcctgac tgtcgtgect ttccaccacg gcttcggaat gttcactact 1680  
ttgggctact ttgcctgagg ttaccggatt gtcacgtta ctaagttcga cgaggagctt 1740  
ttcctgcgca cacttcagga ttacaagtgc actacagtaa tcttgggtgc gacactgttc 1800  
gcaattotta ataggtctga gctccttgat aagtttgacc tctctaacct gactgaaata 1860  
gccagcgggtg gtgctccact tgccaaggag atcggcgagg ctggtgcaag aagattcaac 1920  
ctcccaggcg tccggcaggg atatggactc accgagacta ccagtgcctt tatcatcact 1980  
cctaagggcg acgacaagcc gggagccagc ggcaaggctg tgcctctgtt caagggtgaag 2040  
attattgacc tcgataccaa gaaaacggtg ggtgtcaaca gacggggaga aatctgcgtg 2100  
aaaggaccat ctcttatgtt gggatacacg aacaatcctg aagccaccag agaaactatt 2160  
gacgaggaag gctggctgca cacgggtgac atcgggtact acgacgagga tgagcacttc 2220



-continued

---

tttatagtcg	accgcctgaa	atctctcatt	aagtataaag	gataccaagt	gccaccagct	2280
gaactggagt	ctgtgctcct	gcaacaccct	aacattagag	atgctggtgt	ggccgggggt	2340
cccacacgcg	aggcaggcga	gctgcctgga	gccgtcgttg	tgatggaaaa	gggaaagaca	2400
atgactgaga	aagaaatcgt	agactatgta	aactcccagg	tggtcaacca	caagcggctg	2460
aggggcggcg	tgcggttcgt	agatgaagtc	cccaaggggc	tcacaggaaa	gatcgacgcg	2520
aaagttatca	gggagatact	caagaaacct	caagcaggtg	ggtagtctag	atctagaaat	2580
aattcttact	gtcatgccaa	gtaagatgct	tttctgtgct	gcaatagcag	gcatgctggg	2640
gatgcggtgg	gctctatggc	ttctgaggcg	gaaagaacca	gctggggctc	taggggggat	2700
ccccacgcgc	cctgtagcgg	cgcattaagc	gcggcgggtg	tggtggttac	gcgcagcgtg	2760
accgctacac	ttgccagcgc	cctagcgcgc	gctcctttcg	ctttcttccc	ttcctttctc	2820
gccacgttcg	ccggctttcc	ccgtcaagct	ctaaatcggg	gcatcccttt	agggttcgga	2880
tttagtgctt	tacggcacct	cgaccccaaa	aaacttgatt	agggtgatgg	ttcacgtagt	2940
gggccatcgc	cctgatagac	ggtttttcgc	cctttgacgt	tgtagtccac	gttctttaat	3000
agtggactct	tgttccaaac	tggaacaaca	ctcaacccta	tctcggctca	ttcttttgat	3060
ttataagggg	ttttggggat	ttcggcctat	tggttaaaaa	atgagctgat	ttaacaaaaa	3120
tttaacgcga	attaattctg	tggaatgtgt	gtcagttagg	gtgtggaaag	tccccaggct	3180
ccccaggcag	gcagaagtat	gcaaagcatg	catctcaatt	agtcagcaac	caggtgtgga	3240
aagtccccag	gctccccagc	aggcagaagt	atgcaaagca	tgcactctca	ttagtcagca	3300
accatagtcc	cgcccctaac	tccgcccata	ccgcccctaa	ctccgcccag	ttccgcccata	3360
tctccgcccc	atggctgact	aatttttttt	atztatgcag	aggccgaggc	cgctctgccc	3420
tctgagctat	tccagaagta	gtgaggaggc	ttttttggag	gcctaggctt	ttgcaaaaag	3480
ctcccgggag	cttgtatata	catttttcgga	tctgatcaag	agacaggatg	aggatcgttt	3540
cgcatgattg	aacaagatgg	attgcacgca	ggtctctccg	ccgcttgggt	ggagaggcta	3600
ttcggctatg	actgggcaca	acagacaatc	ggctgctctg	atgccgcctg	gttccggctg	3660
tcagcgcagg	ggcgcgccgt	tctttttgtc	aagaccgacc	tgtccggtgc	cctgaatgaa	3720
ctgcaggacg	aggcagcgcg	gctatcgtgg	ctggccacga	cgggcgttcc	ttgcgcagct	3780
gtgctcgacg	ttgtcactga	agcgggaagg	gactggctgc	tattgggcca	agtgccgggg	3840
caggatctcc	tgtcatctca	ccttgctcct	gccgagaaag	tatccatcat	ggctgatgca	3900
atgcggcggc	tgcatacgct	tgatccggct	acctgcccata	tcgaccacca	agcgaaacata	3960
cgcatcgagc	gagcacgtac	toggatggaa	gccggtcttg	tcgatcagga	tgatctggac	4020
gaagagcata	aggggctcgc	gccagccgaa	ctgttcgcca	ggctcaaggc	gcgcatgccc	4080
gacggcgagg	atctcgtcgt	gacctatggc	gatgcctgct	tgccgaatat	catggtggaa	4140
aatggccgct	tttctggatt	catcgactgt	ggccggctgg	gtgtggcgga	ccgctatcag	4200
gacatagcgt	tggctaccgg	tgatattgct	gaagagcttg	gcggcgaatg	ggctgaccgc	4260
ttcctcgtgc	tttacggtat	cgccgctccc	gattcgcagc	gcatcgctt	ctatcgctt	4320
cttgacgagt	tcttctgagc	gggactctgg	ggttcgaaat	gaccgaccaa	gcgacgccc	4380
acctgccata	acgagatttc	gattccaccg	ccgcttcta	tgaagggttg	ggcttcggaa	4440
tcgttttccg	ggacgccggc	tggatgatcc	tccagcggcg	ggatctcatg	ctggagttct	4500
tcgcccaccc	caacttgttt	attgcagctt	ataatggtta	caaataaagc	aatagcatca	4560

-continued

---

caaatttcac	aaataaagca	tttttttcac	tgcattctag	ttgtggtttg	tccaaactca	4620
tcaatgtatc	ttatcatgtc	tgtataccgt	cgacctctag	ctagagcttg	gcgtaatcat	4680
ggcatagct	gtttcctgtg	tgaattgtt	atccgctcac	aattccacac	aacatacgag	4740
ccggaagcat	aaagtgtaaa	gcctgggggtg	cctaatgagt	gagctaactc	acattaattg	4800
cgttgcgctc	actgcccgtc	ttccagtcgg	gaaacctgtc	gtgccagctg	cattaatgaa	4860
tcgccaacg	cgcggggaga	ggcggtttgc	gtattggcg	ctcttcgct	tcctcgctca	4920
ctgactcgct	gcgctcggtc	gttcggctgc	ggcgagcgg	atcagctcac	tcaaaggcgg	4980
taatacgggt	atccacagaa	tcaggggata	acgcaggaaa	gaacatgtga	gcaaaaggcc	5040
agcaaaaggc	caggaaccgt	aaaaaggccg	cgttgctggc	gtttttccat	aggctccgcc	5100
cccctgacga	gcatcacaaa	aatcgacgct	caagtacagag	gtggcgaaac	ccgacaggac	5160
tataaagata	ccaggcggtt	ccccctggaa	gctccctcgt	gcgctctcct	gttccgacct	5220
tgcgcttac	cggatacctg	tcgcctttc	tccttcggg	aagcgtggcg	ctttctcaat	5280
gctcacgctg	taggtatctc	agttcgggtg	aggctcgttc	ctccaagctg	ggctgtgtgc	5340
acgaaccccc	cgttcagccc	gaccgctgcg	ccttatccgg	taactatcgt	cttgagtcca	5400
acccggtaag	acacgactta	tcgccactgg	cagcagccac	tggtaacagg	attagcagag	5460
cgaggatgt	aggcgggtgct	acagagttct	tgaagtggtg	gcctaactac	ggctacacta	5520
gaaggacagt	atgtgtatc	tgcgctctgc	tgaagccagt	taccttcgga	aaaagagttg	5580
gtagctcttg	atccggcaaa	caaaccaccg	ctggtagcgg	tggttttttt	gtttgcaagc	5640
agcagattac	gcgagaaaa	aaaggatctc	aagaagatcc	tttgatcttt	tctacggggt	5700
ctgacgctca	gtggaacgaa	aactcacggt	aagggatttt	ggcatgaga	ttatcaaaaa	5760
ggatcttcac	ctagatcctt	ttaaattaa	aatgaagttt	taaatcaatc	taaagtatat	5820
atgagtaaac	ttggtctgac	agttaccaat	gcttaatcag	tgaggcacct	atctcagcga	5880
tctgtctatt	tcgttcaccc	atagttgcct	gactccccgt	cgtgtagata	actacgatac	5940
gggagggctt	accatctggc	cccagtgctg	caatgatacc	gcgagacca	cgctcaccgg	6000
ctccagattt	atcagcaata	aaccagccag	ccggaagggc	cgagcgcaga	agtggctcctg	6060
caactttatc	cgctccatc	cagtctatta	attgttgccg	ggaagctaga	gtaagtagtt	6120
cgccagttaa	tagtttgccg	aacgttggtg	ccattgctac	aggcatcgtg	gtgtcacgct	6180
cgctgtttg	tatggcttca	ttcagctccg	gttcccaacg	atcaaggcga	gttacatgat	6240
ccccatggt	gtgcaaaaa	gcggttagct	ccttcggctc	tccgatcgtt	gtcagaagta	6300
agttggccgc	agtgttatca	ctcatgggta	tggcagcact	gcataattct	cttactgtca	6360
tgccatccgt	aagatgcttt	tctgtgactg	gtgagtactc	aaccaagtca	ttctgagaat	6420
agtgatgcg	gcgaccgagt	tgctcttgcc	cggcgtcaat	acgggataat	accgcgccac	6480
atagcagaac	tttaaaagtg	ctcatcattg	gaaaacgttc	ttcggggcga	aaactctcaa	6540
ggatcttacc	gctggtgaga	tcagttcga	tgtaaaccac	tcgtgcaccc	aactgatctt	6600
cagcatcttt	tactttcacc	agcgtttctg	ggtgagcaaa	aacaggaagg	caaatgccg	6660
caaaaaagg	aataaggcg	acacggaaat	gttgaatact	catactcttc	ctttttcaat	6720
attattgaag	catttatcag	ggttattgtc	tcatgagcgg	atacatattt	gaatgtattt	6780
agaaaaataa	acaaatagg	gttccgcgca	catttccccg	aaaagtgcc	cctgacgctc	6839

&lt;210&gt; SEQ ID NO 3

&lt;211&gt; LENGTH: 6833



-continued

---

<212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: A mammalian expression vector expressing Red emitting firefly luciferase (human codon optimized signal) under control of the CMV promoter)

<400> SEQUENCE: 3

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg	60
ccgcatagtt aagccagtat ctgctccctg cttgtgtggt ggaggtcgct gagtagtgcg	120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc	180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt	240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata	300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc	360
cccgccatt gacgtcaata atgacgtatg ttcccattag aacgccaata gggactttcc	420
atgacgtca atgggtggac tatttacggg aaactgcccc cttggcagta catcaagtgt	480
atcatatgcc aagtagcccc cctattgacg tcaatgacgg taaatggccc gcctggcatt	540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca	600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggctgga tagcggtttg	660
actcacgggg atttccaagt ctccaccccc ttgacgtcaa tgggagtttg ttttggcacc	720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg	780
gtaggcgtgt acgggtggag gtctatataa gcagagctct ctggctaact agagaacca	840
ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gcttgggtacc	900
gagctcggat ccatggaaac agaaagagaa gaaaacgttg tctacggccc actgccattc	960
taccgatcg aggagggtc tgccggcatc caattgcaca agtacatgca acaatagccc	1020
aagctcggcg ccatcgctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag	1080
tacttcgaca tcacgtgcag actcggcgag gctatgaaga actacggcat gaagccagaa	1140
ggacacatcg ctctctgtag cgagaactgc gaagagttct tcattcctgt tctggctggt	1200
ctttacatcg gagttacagt cgcgccaaact aacgaaattt atacacttag agagctgaac	1260
cacagtctgg ggatagccca acctactatc gtattctcta gcaggaaggg cctgccc aaa	1320
gtgcttgagg tgcagaagac cgtgacttgc atcaaaaacca ttgtcatcct ggacagtaag	1380
gtcaacttcg gcggttatga ctgcgtagag accttcatta agaaacacgt cgagctgggc	1440
tttctgcca cctcatttgt gcccacgac gtcaaagacc ggaagcacca cattgctctg	1500
cttatgaact cttccggttc cacagggtc cccaaaggag tagagatcac tcacgaggcc	1560
ctggtcacga gattctctca cgctaaggac cctatatacg gcaatcaggt ggccccaggt	1620
accgctatcc tgactgtcgt gcctttccac cacggcttcg gaatgttcac tactttgggc	1680
tactttgect gcggttaccg gattgtcatg ctactaagt tcgacgagga gcttttctg	1740
cgcacacttc aggattacaa gtgcactaca gtaatcctgg tgccgacact gttcgcaatt	1800
cttaataggt ctgagctcct tgataagttt gacctctcta acctgactga aatagccagc	1860
ggtggtgctc cacttgccaa ggagatcggc gaggtgttg caagaagatt caacctccca	1920
ggcgtccggc agggatatgg actcacggag actaccagtg cctttatcat cactcctaag	1980
ggcgacgaca agccgggagc cagcggcaag gtcgtgcctc tgttcaaggt gaagattatt	2040
gacctcgata ccaagaaaac gttgggtgtc aacagacggg gagaaatctg cgtgaaagga	2100

-continued

---

ccatctctta	tggtgggata	cacgaacaat	cctgaagcca	ccagagaaac	tattgacgag	2160
gaaggctggc	tgacacacggg	tgacatcggg	tactacgacg	aggatgagca	cttctttata	2220
gtcgaccgcc	tgaaatctct	cattaagtat	aaaggatacc	aagtgccacc	agctgaactg	2280
gagtctgtgc	tcctgcaaca	ccctaacatt	agagatgctg	gtgtggccgg	ggttccccgac	2340
agcgaggcag	gcgagctgcc	tggagccgtc	gttgtgatgg	aaaagggaaa	gacaatgact	2400
gagaaagaaa	tcgtagacta	tgtaaacctc	caggtggtca	accacaagcg	gctgagggggc	2460
ggcgtgcggt	tcgtagatga	agtccccaa	gggctcacag	gaaagatcga	cgcgaaagtt	2520
atcagggaga	tactcaagaa	acctcaagca	ggtgggtagt	ctagatctag	aaataattct	2580
tactgtcatg	ccaagtaaga	tgcttttctg	tgctgcaata	gcaggcatgc	tggggatgcg	2640
gtgggctcta	tggcttctga	ggcggaaaga	accagctggg	gctctagggg	gtatccccac	2700
gcgccctgta	gcgccgcatt	aagcgcggcg	ggtgtggtgg	ttacgcgcag	cgtagaccgt	2760
acacttgcca	gccccctagc	gccccctcct	ttcgctttct	tcccttcctt	tctcgccacg	2820
ttcgccggct	ttccccgtca	agctctaaat	cgggcatcc	cttaggggtt	ccgatttagt	2880
gctttacggc	acctcgacc	caaaaaactt	gattagggtg	atggttcacg	tagtgggcca	2940
tcgccctgat	agacggtttt	tcgccctttg	acgttgaggt	ccacgttctt	taatagtgga	3000
ctcttgttcc	aaactggaac	aacactcaac	cctatctcgg	tctattcttt	tgatttataa	3060
gggattttgg	ggatttcggc	ctattgggta	aaaaatgagc	tgatttaaca	aaaatttaac	3120
gcgaattaat	tctgtggaat	gtgtgtcagt	taggggtggt	aaagtcccc	ggctccccag	3180
gcaggcagaa	gtatgcaaag	catgcatctc	aattagtcag	caaccaggtg	tggaaagtcc	3240
ccaggctccc	cagcaggcag	aagtatgcaa	agcatgcate	tcaattagtc	agcaaccata	3300
gtcccgeccc	taactccgcc	catcccgecc	ctaactccgc	ccagttccgc	ccattctccg	3360
ccccatggct	gactaatttt	ttttatttat	gcagaggccg	aggccgcctc	tgcctctgag	3420
ctattccaga	agtagtgagg	aggctttttt	ggaggcctag	gcttttgcaa	aaagctcccg	3480
ggagcttgta	tatccatttt	cggatctgat	caagagacag	gatgaggatc	gtttcgcag	3540
attgaacaag	atggattgca	cgcaggttct	ccggccgctt	gggtggagag	gctattcggc	3600
tatgactggg	cacaacagac	aatcggctgc	tctgatgccg	ccgtgttccg	gctgtcagcg	3660
caggggcgcc	cggttctttt	tgtcaagacc	gacctgtccg	gtgccctgaa	tgaactgcag	3720
gacgaggcag	cgcggtatc	gtggctggcc	acgacggcg	ttccttgccg	agctgtgctc	3780
gacgttgca	ctgaagcggg	aaggactgg	ctgctattgg	gcgaagtgcc	ggggcaggat	3840
ctcctgtcat	ctcaccttgc	tcctgccgag	aaagtatcca	tcatggctga	tgcaatgcgg	3900
cggctgcata	cgcttgatcc	ggctacctgc	ccattcgacc	accaagcgaa	acatcgcatc	3960
gagcagcac	gtactcggat	ggaagccgg	cttgtcgatc	aggatgatct	ggacgaagag	4020
catcaggggc	tcgcgccagc	cgaactgttc	gccaggctca	aggcgcgcag	gcccgcggc	4080
gaggatctcg	tcgtgacca	tggcgatgcc	tgettgcga	atatcatggt	ggaaaatggc	4140
cgcttttctg	gattcatcga	ctgtggccgg	ctgggtgtgg	cggaccgcta	tcaggacata	4200
gcgttggtta	cccgtgatat	tgctgaagag	cttggcggcg	aatgggctga	ccgcttctc	4260
gtgctttacg	gtatcgccgc	tcccgatctg	cagcgcacgc	ccttctatcg	ccttcttgac	4320
gagttcttct	gagcgggact	ctggggttcg	aaatgaccga	ccaagegacg	cccaacctgc	4380
catcacgaga	tttcgattcc	accgccgctt	tctatgaaag	gttgggcttc	ggaatcgttt	4440
tccgggacgc	cggctggatg	atcctccagc	gcggggatct	catgctggag	ttcttcgccc	4500



-continued

---

acccaactt	gtttattgca	gcttataatg	gttaciaaata	aagcaatagc	atcaciaaatt	4560
tcaciaaataa	agcatttttt	tcaactgcatt	ctagttgtgg	tttgtccaaa	ctcatcaatg	4620
tatcttatca	tgtctgtata	cogtcgacct	ctagctagag	cttggegtaa	tcatggcat	4680
agctgtttcc	tgtgtgaaat	tgttatcegc	tcaciaattcc	acaciaacata	cgagccggaa	4740
gcataaagtg	taaagcctgg	ggtgcctaata	gagtgagcta	actcacatta	attgcgttgc	4800
gctcaactgcc	cgctttccag	tcgggaaacc	tgctgtgcca	gctgcattaa	tgaatcggcc	4860
aacgcgctgg	gagagcggt	ttgcgtattg	ggcgtcttc	cgcttctctg	ctcaactgact	4920
cgctgcgctc	ggcgttccg	ctgcggcgag	cggtatcagc	tcaactcaaag	gcggaatac	4980
ggttatccac	agaatcaggg	gataacgcag	gaaagaacat	gtgagcaaaa	ggccagcaaa	5040
aggccaggaa	ccgtaaaaag	gcccgcgttgc	tggcgttttt	ccataggctc	cgccccctg	5100
acgagcatca	caaaaatcga	cgctcaagtc	agaggtggcg	aaaccgcaca	ggactataaa	5160
gataaccaggc	gtttccccct	ggaagctccc	tcgtgcgctc	tctgttccg	acctgcccgc	5220
ttaccggata	cctgtccgcc	ttctccctt	cggaagcgt	ggcgtttct	caatgctcac	5280
gctgtaggta	tctcagttcg	gtgtaggtcg	ttcgtccaa	gctgggctgt	gtgcacgaac	5340
ccccggtca	gcccgaccgc	tgcccttat	ccgtaacta	tcgtcttgag	tccaaccgg	5400
taagacacga	cttatcgcca	ctggcagcag	ccactggtaa	caggattagc	agagcgaggt	5460
atgtaggcgg	tgctacagag	ttcttgaagt	ggtggcctaa	ctacggctac	actagaagga	5520
cagtatttgg	tatctgcgct	ctgctgaagc	cagttacct	cggaaaaaga	gttggtagct	5580
cttgatccgg	caaaaaacc	accgctggta	gcggtggttt	ttttgtttgc	aagcagcaga	5640
ttaccgagcag	aaaaaaagga	tctcaagaag	atcctttgat	cttttctacg	gggtctgacg	5700
ctcagtggaa	cgaaaactca	cgtaaggga	ttttggtcat	gagattatca	aaaaggatct	5760
tcacctagat	ccttttaaat	taaaaatgaa	gttttaaatc	aatctaaagt	atatatgagt	5820
aaacttggtc	tgacagttac	caatgcttaa	tcagtgaggc	acctatctca	gcgatctgtc	5880
tatttcggtc	atccatagtt	gcctgactcc	ccgtcgtgta	gataactacg	atacgggagg	5940
gcttaccatc	tggccccagt	gctgcaatga	taccgcgaga	cccacgctca	ccggctccag	6000
atztatcagc	aataaaccag	ccagccggaa	gggcccagcg	cagaagtgg	cctgcaactt	6060
tatccgctc	catccagtct	attaattggt	gccgggaagc	tagagtaagt	agttcgccag	6120
ttaatagttt	gcgcaacggt	gttgccattg	ctacaggcat	cgtgggtgca	cgctcgtcgt	6180
ttggtatggc	ttcattcagc	tccggttccc	aacgatcaag	gcgagttaca	tgatccccc	6240
tggtgtgcaa	aaaagcgtt	agtccttcg	gtcctccgat	cgttgtcaga	agtaagttgg	6300
ccgcagtgtt	atcaactcatg	gttatggcag	caactgcataa	ttctcttact	gtcatgcat	6360
ccgtaagatg	cttttctgtg	actggtgagt	actcaaccaa	gtcattctga	gaatagtgt	6420
tgccgagacc	gagttgctct	tgcccggcgt	caatacggga	taataccgcg	ccacatagca	6480
gaactttaa	agtgtctatc	attggaaaac	gttcttcggg	gcgaaaactc	tcaaggatct	6540
taccgctgtt	gagatccagt	tcgatgtaac	ccactcgtgc	acccaactga	tcttcagcat	6600
cttttacttt	caccagcgtt	tctgggtgag	caaaaacagg	aaggcaaaat	gcccgaaaaa	6660
aggaataag	ggcgacacgg	aaatggtgaa	tactcactact	cttcttttt	caatattatt	6720
gaagcattta	tcagggttat	tgtctcatga	gcggatacat	atgtgaatgt	attagaaaa	6780
ataaacaat	aggggttccg	cgcacatttc	cccgaaggt	gccacctgac	gtc	6833

-continued

---

```

<210> SEQ ID NO 4
<211> LENGTH: 6827
<212> TYPE: DNA
<213> ORGANISM: Luciola italica
<220> FEATURE:
<221> NAME/KEY: CMV promoter bases
<222> LOCATION: (209) .. (863)
<220> FEATURE:
<221> NAME/KEY: Green emitting firefly luciferase gene
<222> LOCATION: (907) .. (2560)
<220> FEATURE:
<221> NAME/KEY: T7 promoter bases
<222> LOCATION: (1827) .. (1845)
<220> FEATURE:
<221> NAME/KEY: Polylinker bases
<222> LOCATION: (1852) .. (1870)
<220> FEATURE:
<221> NAME/KEY: Synthetic polyadenylation site
<222> LOCATION: (2560) .. (2604)
<220> FEATURE:
<221> NAME/KEY: SP6 promoter
<222> LOCATION: (2576) .. (2593)
<220> FEATURE:
<221> NAME/KEY: SV40 promoter bases
<222> LOCATION: (3145) .. (3480)
<220> FEATURE:
<221> NAME/KEY: SV40 origin of replication: bases
<222> LOCATION: (3259) .. (3344)
<220> FEATURE:
<221> NAME/KEY: Neomycin ORF bases
<222> LOCATION: (3516) .. (4310)
<220> FEATURE:
<221> NAME/KEY: ColE1 origin: bases
<222> LOCATION: (3934) .. (4607)
<220> FEATURE:
<221> NAME/KEY: SV40 PolyA: bases
<222> LOCATION: (4365) .. (4737)
<220> FEATURE:
<221> NAME/KEY: Ampicillin ORF: bases
<222> LOCATION: (4752) .. (5612)

<400> SEQUENCE: 4

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg      60
ccgcatagtt aagccagtat ctgctccctg cttgtgtggt ggaggtcgct gagtagtgcg      120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc      180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt      240
gattattgac tagttattaa tagtaatcaa ttacgggggc attagttcat agcccatata      300
tggagtcccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc      360
cccgccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc      420
attgacgtca atgggtggac tatttacggt aaactgcca cttggcagta catcaagtgt      480
atcatatgcc aagtacgcc cctattgacg tcaatgacgg taaatggccc gcctggcatt      540
atgccagta catgacctta tgggactttc ctacttgga gtacatctac gtattagtca      600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg      660
actcacgggg atttccaagt ctccaccca ttgacgtcaa tgggagtttg ttttggcacc      720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc ccattgacg caaatgggcg      780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca      840
ctgcttactg gcttatcgaa attaatacga ctactatag ggagaccaa gcttgggtacc      900
gagctcggat ccatggaac agaaagagaa gaaaacgctt tctacggccc actgccatc      960
taccgatcg aggagggtc tgccggcatc caattgcaca agtacatgca acaatcggc      1020
aagctcggcg ccatcgcctt cagtaacgcc ctgacaggcg tcgacatcag ctaccagcag      1080

```



-continued

---

tacttcgaca	tcacgtgcag	actcgccgag	gctatgaaga	actacggcat	gaagccagaa	1140
ggacacatcg	ctctctgtag	cgagaactgc	gaagagttct	tcattcctgt	tctggctggt	1200
ctttacatcg	gagttacagt	cgcgccaact	aacgaaattt	atacacttag	agagctgaac	1260
cacagtctgg	ggatagccca	acctactatc	gtattctcta	gcaggaagg	cctgccc aaa	1320
gtgcttgagg	tgcaagaagac	cgtgacttgc	atcaaaaacca	ttgtcatcct	ggacagtaag	1380
gtcaacttcg	gcggttatga	ctgcgtagag	accttcatta	agaaacacgt	cgagctgggc	1440
tttctgcca	cctcatttgt	gcccacgac	gtcaaaagacc	ggaagcacca	cattgctctg	1500
cttatgaact	cttccggttc	cacagggctg	cccaaaggag	tagagatcac	tcacgaggcc	1560
ctggtcacga	gattctctca	cgctaaggac	cctatatacg	gcaatcaggt	ggccccaggt	1620
accgctatcc	tgactgtcat	ccctttccac	cacgccttcg	gaatgagcac	tactttgggc	1680
tactttgctt	gcggttacgg	gattgtcatg	cttactaagt	tcgacgagga	gcttttctctg	1740
cgcacacttc	aggattacaa	gtgcactagc	gtaatcctgg	tgccgacact	gttcgcaatt	1800
cttaataggt	ctgagctcct	tgataagttt	gacctctcta	acctgactga	aatagccagc	1860
ggtggtgctc	cacttgccaa	ggagatcggc	gaggctgttg	caagaagatt	caacctccca	1920
ggcgtccggc	agggatatgg	actcaccgag	actaccagtg	cctttatcat	cactcctaag	1980
ggcgacgaca	agccgggagc	cagcggcaag	gtcgtgcctc	tgttcaaggt	gaagattatt	2040
gacctcgata	ccaagaaaac	gttgggtgtc	aacagacggg	gagaaatctg	cgtgaaagga	2100
ccatctctta	tgttgggata	cacgaacaat	cctgaagcca	ccagagaaac	tattgacgag	2160
gaaggctggc	tgacacacggg	tgacatcggg	tactacgacg	aggatgagca	cttctttata	2220
gtcgaccgcc	tgaaatctct	cattaagtat	aaaggatacc	aagtgccacc	agctgaactg	2280
gagtctgtgc	tcctgcaaca	ccctaacatt	agagatgctg	gtgtggccgg	ggttccccgac	2340
agcgaggcag	gcgagctgcc	tggagccgtc	gttgtgatgg	aaaagggaaa	gacaatgact	2400
gagaaagaaa	tcgtagacta	tgtaaaactcc	caggtggtca	accacaagcg	gctgagggggc	2460
ggcgtgcggg	tcgtagatga	agtccccaa	gggtccacag	gaaagatcga	cgcgaaagtt	2520
atcagggaga	tactcaagaa	acctcaagca	ggtgggtagt	ctagaaataa	ttcttactgt	2580
catgccaaagt	aagatgcttt	tctgtgctgc	aatagcaggc	atgctgggga	tgcgggtgggc	2640
tctatggctt	ctgaggcggg	aagaaccagc	tggggctcta	gggggtatcc	ccacgcgccc	2700
tgtagcggcg	cattaagcgc	ggcgggtgtg	gtggttacgc	gcagcgtgac	cgctacactt	2760
gccagcgccc	tagecggcgc	tcctttcget	ttcttccctt	cctttctcgc	caegtctgcc	2820
ggctttcccc	gtcaagctct	aaatcggggc	atccctttag	ggttccgatt	tagtgcttta	2880
cggcacctcg	acccccaaaa	acttgattag	ggtgatgggt	cacgtagtgg	gccatcgccc	2940
tgatagacgg	tttttcgccc	tttgacgttg	gagtccacgt	tctttaatag	tggactcttg	3000
ttccaaactg	gaacaacact	caacctatc	tcggtctatt	cttttgattt	ataagggatt	3060
ttggggattt	cggcctattg	gttaaaaaat	gagctgattt	aacaaaaatt	taacgcgaat	3120
taattctgtg	gaatgtgtgt	cagttagggt	gtggaaagtc	cccaggctcc	ccaggcaggc	3180
agaagtatgc	aaagcatgca	tctcaattag	tcagcaacca	ggtgtggaaa	gtccccaggc	3240
tccccagcag	gcagaagtat	gcaaagcatg	catctcaatt	agtcagcaac	catagtcccc	3300
cccctaactc	cgccccatccc	gcccctaact	ccgcccagtt	ccgcccattc	tccgccccat	3360
ggctgactaa	ttttttttat	ttatgcagag	gccgaggccg	cctctgcctc	tgagctattc	3420

-continued

---

cagaagtagt	gaggaggctt	ttttggagge	ctaggctttt	gcaaaaagct	cccgggagct	3480
tgtatatcca	ttttcggatc	tgatcaagag	acaggatgag	gatcgtttcg	catgattgaa	3540
caagatggat	tgcacgcagg	ttctccggcc	gcttggggtg	agaggctatt	cggtatgac	3600
tgggcacaac	agacaatcgg	ctgctctgat	gccgccgtgt	tccggctgtc	agcgcagggg	3660
cgcccggttc	tttttgtcaa	gaccgacctg	tccggtgccc	tgaatgaact	gcaggacgag	3720
gcagcggggc	tatcgtggct	ggccacgacg	ggcgttcctt	gcgcagctgt	gctcgcgctt	3780
gtcactgaag	cggaaggga	ctggctgcta	ttgggcgaag	tgccggggca	ggatctcctg	3840
tcatctcacc	ttgctcctgc	cgagaaagta	tccatcatgg	ctgatgcaat	gcggcggctg	3900
catacgcttg	atccggctac	ctgcccattc	gaccaccaag	cgaaacatcg	catcgagcga	3960
gcacgtactc	ggatggaagc	cggtcttgtc	gatcaggatg	atctggacga	agagcatcag	4020
gggctcgcgc	cagccgaact	gttcgccagg	ctcaaggcgc	gcatgcccca	cggcgaggat	4080
ctcgtcgtga	cccattggcg	tgctcgttgc	ccgaatatca	tggtggaaaa	tggccgcttt	4140
tctggattca	tgcactgtgg	ccggctgggt	gtggcggacc	gctatcagga	catagcgttg	4200
gctaccgctg	atattgctga	agagcttggc	ggcgaatggg	ctgaccgctt	cctcgtgctt	4260
tacggtatcg	ccgctcccga	ttcgcagcgc	atcgccttct	atcgccttct	tgacgagttc	4320
ttctgagcgg	gactctgggg	ttcgaaatga	ccgaccaagc	gacgcccac	ctgccatcac	4380
gagatttcga	ttccaccgcc	gccttctatg	aaaggttggg	cttcggaatc	gttttccggg	4440
acgccggctg	gatgatcctc	cagcgcgggg	atctcatgct	ggagttcttc	gcccacccca	4500
acttgtttat	tgcagcttat	aatggttaca	aataaagcaa	tagcatcaca	aatttcacaa	4560
ataaagcatt	tttttactg	cattctagtt	gtggtttgtc	caaactcatc	aatgtatctt	4620
atcatgtctg	tataccgctg	acctctagct	agagcttggc	gtaatcatgg	tcatagctgt	4680
ttcctgtgtg	aaattgttat	ccgctcacia	ttccacacia	catacgagcc	ggaagcataa	4740
agtgtaaagc	ctggggtgcc	taatgagtga	gctaactcac	attaattgcg	ttgcgctcac	4800
tgcccgcttt	ccagtcggga	aacctgtcgt	gccagctgca	ttaatgaatc	ggccaacgcg	4860
cggggagagg	cggtttgctg	attgggcgct	cttcgcttc	ctcgtcact	gactcgtgct	4920
gctcggctcg	tccgctgctg	cgagcgggat	cagctcactc	aaaggcggta	atacggttat	4980
ccacagaatc	aggggataac	gcaggaaaga	acatgtgagc	aaaaggccag	caaaaggcca	5040
ggaaccgtaa	aaaggccgcg	ttgctggcgt	ttttccatag	gctccgcccc	cctgacgagc	5100
atcacaaaaa	tgcagctca	agtcagaggt	ggcgaacccc	gacaggacta	taaagatacc	5160
aggcgtttcc	ccctggaagc	tccctcgtgc	gctctcctgt	tccgaccctg	ccgcttaccg	5220
gatacctgct	cgcctttctc	ccttcgggaa	gcgtggcgt	ttctcaatgc	tcaagctgta	5280
ggtatctcag	ttcgggtgag	gtcgttcgct	ccaagctggg	ctgtgtgcac	gaaccccccg	5340
ttcagcccga	ccgctgcgcc	ttatccggta	actatcgtct	tgagtccaac	ccggttaagac	5400
acgacttata	gccactggca	gcagccactg	gtaacaggat	tagcagagcg	aggatgtag	5460
gcgggtgctac	agagttcttg	aagtgggtggc	ctaactacgg	ctacactaga	aggacagtat	5520
ttggtatctg	cgctctgctg	aagccagtta	ccttcggaaa	aagagttggt	agctcttgat	5580
ccggcaaaaca	aaccaccgct	ggtagcggtg	gtttttttgt	ttgcaagcag	cagattacgc	5640
gcagaaaaaaa	aggatctcaa	gaagatcctt	tgatcttttc	tacggggtct	gacgctcagt	5700
ggaacgaaaa	ctcacgttaa	gggattttgg	tcatgagatt	atcaaaaagg	atcttcacct	5760
agatcctttt	aaattaaaaa	tgaagtttta	aatcaatcta	aagtatatat	gagtaaactt	5820



-continued

---

```

ggctctgacag ttaccaatgc ttaatcagtg aggcacctat ctcagcgatc tgtctatttc 5880
gttcatccat agttgectga ctccccgctg ttagataaac tacgatacgg gagggcttac 5940
catctggccc cagtgcctga atgataccgc gagaccacg ctcaccggct ccagatttat 6000
cagcaataaa ccagccagcc ggaagggccg agcgcagaag tggctctgca actttatccg 6060
cctccatcca gtctattaat tgttgccggg aagctagagt aagtagttcg ccagttaata 6120
gtttgcgcaa cgttgttgcc attgctacag gcatcgtggg gtcacgctcg tcgtttggta 6180
tggcttcatt cagctccggg tcccacgat caaggcgagt tacatgatcc cccatgttgt 6240
gcaaaaaagc ggtagctcc ttcggctctc cgatcgttgt cagaagtaag ttggccgag 6300
tgttatcact catggttatg gcagcactgc ataattctct tactgtcatg ccatccgtaa 6360
gatgcttttc tgtgactggg gagtactcaa ccaagtcatt ctgagaatag tgtatgcggc 6420
gaccgagttg ctcttgcccg gcgtcaatac gggataatac cgcgccacat agcagaactt 6480
taaaagtgct catcattgga aaacgttctt cggggcgaaa actctcaagg atcttaccgc 6540
tgttgagatc cagttcgatg taaccactc gtgcacccaa ctgatcttca gcatctttta 6600
ctttcaccag cgtttctggg tgagcaaaaa caggaaggca aatgccgca aaaaggaa 6660
taaggcgac acggaaatgt tgaatactca tactcttctt tttcaatat tattgaagca 6720
tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag aaaataaac 6780
aaataggggt tccgcgcaca tttccccgaa aagtgccacc tgacgctc 6827

```

&lt;210&gt; SEQ ID NO 5

&lt;211&gt; LENGTH: 6834

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Renilla reniformis

&lt;400&gt; SEQUENCE: 5

```

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtggt ggaggtcgtc gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatagc cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
cccgccatt gacgtcaata atgacgatg tcccacatg aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggg aaactgccc cttggcagta catcaagtgt 480
atcatatgcc aagtacgcc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgccagta catgacctt tgggactttc ctacttgcca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660
actcacgggg atttccaagt ctccaccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc ccattgacg caaatgggcg 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaacca 840
ctgcttactg gcttatcga attaatacga ctactatag ggagaccaa gcttggtacc 900
gagctcggat ccagccacca tggaaacaga aagagaagaa aacgttgtct acggcccact 960
gccattctac ccgatcgagg agggctctgc cggcatccaa ttgcacaagt acatgcaaca 1020
atagccaag ctcggcgcca tcgccttcag taacgcctg acaggcgtcg acatcagcta 1080

```

-continued

---

ccagcagtac	ttcgacatca	cgtgcagact	cgccgaggct	atgaagaact	acggcatgaa	1140
gccagaagga	cacatcgctc	tctgtagcga	gaactgcgaa	gagttcttca	ttcctgttct	1200
ggctggctct	tacatcggag	ttacagtcgc	gccaaactaac	gaaatttata	cacttagaga	1260
gctgaaccac	agtctgggga	tagcccaacc	tactatcgta	ttctctagca	ggaagggcct	1320
gccccaaagt	cttgaggtgc	agaagaccgt	gacttgcac	aaaaccattg	tcatcctgga	1380
cagtaaggtc	aacttcggcg	gttatgactg	cgtagagacc	ttcattaaga	aacacgtcga	1440
gctgggcttt	cctgccacct	catttgtgcc	catcgacgtc	aaagaccgga	agcaccacat	1500
tgctctgctt	atgaactctt	ccggttccac	agggtgccc	aaaggagtag	agatcactca	1560
cgaggccctg	gtcacgagat	tctctcacgc	taaggaccct	atatacggca	atcaggtggc	1620
cccaggtacc	gctatcctga	ctgtcgtgcc	tttccaccac	ggcttcggaa	tgttcactac	1680
tttgggctac	tttgectgcg	gttaccggat	tgtcatgctt	actaagtctg	acgaggagct	1740
tttctgctgc	acacttcagg	attacaagtg	cactacagta	atcctggtgc	cgacactggt	1800
cgcaattctt	aataggtctg	agctccttga	taagtttgac	ctctctaacc	tgactgaaat	1860
agccagcggg	gggtgctccac	ttgccaagga	gatcggcgag	gctggtgcaa	gaagattcaa	1920
cctcccaggc	gtccggcagg	gatatggact	caccgagact	accagtgcct	ttatcatcac	1980
tcctaagggc	gacgacaagc	cgggagccag	cggaaggtc	gtgcctctgt	tcaaggtgaa	2040
gattattgac	ctcgatacca	agaaaacggt	gggtgtcaac	agacggggag	aaatctgcgt	2100
gaaaggacca	tctcttatgt	tgggatacac	gaacaatcct	gaagccacca	gagaaactat	2160
tgacgaggaa	ggctggctgc	acacgggtga	catcgggtac	tacgacgagg	atgagcactt	2220
ctttatagtc	gaccgcctga	aatctctcat	taagtataaa	ggataccaag	tgccaccagc	2280
tgaactggag	tctgtgctcc	tgcaacaccc	taacattaga	gatgctggtg	tggccggggg	2340
tcccagacgc	gaggcaggcg	agctgcctgg	agccgtcggt	gtgatggaaa	agggaaagac	2400
aatgactgag	aaagaaatcg	tagactatgt	aaactcccag	gtggtcaacc	acaagcggct	2460
gaggggcggc	gtgcggttcg	tagatgaagt	ccccaaaggg	ctcacaggaa	agatcgacgc	2520
gaaagttatc	agggagatac	tcaagaaacc	tcaagcaggt	gggtagtcta	gaaataattc	2580
ttactgtcat	gccaagtaag	atgcttttct	gtgctgcaat	agcaggcatg	ctgggggatgc	2640
gggtggctct	atggcttctg	aggcggaaa	aaccagctgg	ggctctaggg	ggtatcccca	2700
cgcgccctgt	agcggcgcat	taagcgcggc	gggtgtggtg	gttacgcgca	gcgtgaccgc	2760
tacacttgcc	agcgccttag	cgcgccctcc	tttgccttcc	ttcccttctt	ttctcgccac	2820
gttcgcgggc	tttccccgtc	aagctctaaa	tcggggcacc	cctttagggt	tccgatttag	2880
tgctttacgg	cacctcgacc	ccaaaaaact	tgattagggt	gatggttcac	gtagtgggccc	2940
atcgccctga	tagacggttt	ttcgcccttt	gacgttgag	tccacgttct	ttaatagtgg	3000
actcttgctc	caactggaa	caaacactcaa	ccctatctcg	gtctattctt	ttgatttata	3060
agggattttg	gggatttcgg	cctattgggt	aaaaaatgag	ctgatttaac	aaaaatttaa	3120
cgcgaaattaa	ttctgtggaa	tgtgtgtcag	ttagggtgtg	gaaagtcccc	aggtccccca	3180
ggcaggcaga	agtatgcaa	gcatgcatct	caattagtca	gcaaccaggt	gtggaaagtc	3240
cccaggctcc	ccagcaggca	gaagtatgca	aagcatgcat	ctcaattagt	cagcaaccat	3300
agtcccggcc	ctaactccgc	ccatcccgcc	cctaactccg	cccagttccg	cccattctcc	3360
gccccatggc	tgactaattt	tttttattta	tgcagaggcc	gaggccgcct	ctgcctctga	3420
gctattccag	aagtagtgag	gaggcttttt	tggaggccta	ggcttttgca	aaaagctccc	3480



-continued

---

gggagcttgt	atatccattt	tcggatctga	tcaagagaca	ggatgaggat	cgtttcgcat	3540
gattgaacaa	gatggattgc	acgcaggttc	tccggccgct	tgggtggaga	ggctattcgg	3600
ctatgactgg	gcacaacaga	caatcggtcg	ctctgatgcc	gccgtgttcc	ggctgtcagc	3660
gcagggggcg	cgggttcttt	ttgtcaagac	cgacctgtcc	ggtgccctga	atgaactgca	3720
ggacgaggca	gcgcggctat	cgtggctggc	cacgacgggc	gttccttgcg	cagctgtgct	3780
cgacgttgtc	actgaagcgg	gaagggactg	gctgctattg	ggcgaagtgc	cggggcagga	3840
tctcctgtca	tctcaccttg	ctcctgccga	gaaagtatcc	atcatggctg	atgcaatgcg	3900
gcggctgcat	acgcttgatc	cggctacctg	cccattcgac	caccaagcga	aacatcgcat	3960
cgagcgagca	cgtactcgga	tggaagccgg	tcttgctgat	caggatgatc	tggacgaaga	4020
gcatcagggg	ctcgcgccag	ccgaactggt	cgccaggctc	aaggcgcgca	tgcccgacgg	4080
cgaggatctc	gtcgtgaccc	atggcgatgc	ctgcttgccg	aatatcatgg	tggaaaatgg	4140
ccgcttttct	ggattcatcg	actgtggccg	gctgggtgtg	gcggaccgct	atcaggacat	4200
agcgttggt	accegtgata	ttgctgaaga	gcttgccggc	gaatgggctg	accgcttctt	4260
cgtgctttac	ggtatcgccg	ctcccgatcc	gcagcgcatac	gccttctatc	gccttcttga	4320
cgagttcttc	tgagcgggac	tctggggttc	gaaatgaccg	accaagcgac	gcccacactg	4380
ccatcacgag	atttcgatcc	caccgcccgc	ttctatgaaa	ggttgggctt	cggaatcggt	4440
ttccgggacg	cggctgggat	gatcctccag	cgcggggatc	tcatgctgga	gttcttcgcc	4500
caccccaact	tgtttattgc	agcttataat	ggttacaat	aaagcaatag	catcacaat	4560
ttcacaata	aagcattttt	ttactgcat	tctagtgtg	gtttgtcaa	actcatcaat	4620
gtatcttatac	atgtctgtat	accgtcgacc	tctagctaga	gcttggcgta	atcatggtca	4680
tagctgtttc	ctgtgtgaaa	ttgttatccg	ctcacaattc	cacacaacat	acgagccgga	4740
agcataaagt	gtaaagcctg	gggtgcctaa	tgagtgagct	aactcacatt	aattgcgctg	4800
cgctcactgc	ccgctttcca	gtcgggaaac	ctgtcgtgcc	agctgcatta	atgaatcggc	4860
caacgcgcgg	ggagaggcgg	tttgcgatt	gggcgctctt	ccgcttctc	gctcactgac	4920
tcgctgcgct	cggctgctcg	gctgcggcga	gcggtatcag	ctcactcaa	ggcggtaata	4980
cggttatcca	cagaatcagg	ggataacgca	ggaaagaaca	tgtgagcaaa	aggccagcaa	5040
aaggccagga	accgtaaaaa	ggccgcgctg	ctggcgtttt	tccataggct	ccgccccct	5100
gacgagcatc	acaaaaatcg	acgctcaagt	cagaggtggc	gaaaccgac	aggactataa	5160
agataccagg	cgtttcccc	tggaagctcc	ctcgtgcgct	ctcctgttcc	gacctgccc	5220
cttaccggat	acctgtccgc	ctttctccct	tcgggaagcg	tggcgcttcc	tcaatgctca	5280
cgctgtaggt	atctcagttc	gggtgtaggtc	gttcgctcca	agctgggctg	tgtgcacgaa	5340
cccccgctc	agcccgaccg	ctgcgcctta	tccggttaact	atcgtcttga	gtccaaccg	5400
gtaagacacg	acttatcgcc	actggcagca	gccactggta	acaggattag	cagagcgagg	5460
tatgtaggag	gtgctacaga	gttcttgaag	tgggtggccta	actacggcta	cactagaagg	5520
acagtatttg	gtatctgcgc	tctgctgaag	ccagttacct	tcggaaaaag	agttggtagc	5580
tcttgatccg	gcaacaaac	caccgctggt	agcggtggtt	ttttgtttg	caagcagcag	5640
attacgcgca	gaaaaaaag	atctcaagaa	gatcctttga	tcttttctac	ggggtctgac	5700
gctcagtggg	acgaaaactc	acgttaaggg	atthttggta	tgagattatc	aaaaaggatc	5760
ttcacctaga	tccttttaaa	ttaaaaatga	agttttaaat	caatctaaag	tatatatgag	5820

-continued

---

```

taaacttggg ctgacagtta ccaatgctta atcagtgagg cacctatctc agcgatctgt 5880
ctatttcggt catccatagt tgccctgactc cccgtcgtgt agataactac gatacgggag 5940
ggcttaccat ctggccccag tgctgcaatg ataccgagag acccacgctc accggctcca 6000
gatttatcag caataaacca gccagccgga agggccgagc gcagaagtgg tcctgcaact 6060
ttatccgctt ccatccagtc tattaattgt tgccgggaag ctagagtaag tagttcgcca 6120
gtaaatagtt tgcgcaacgt tgttgccatt gctacaggca tcgtggtgtc acgctcgtcg 6180
tttggtatgg cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatcccc 6240
atggttgca aaaaagcggg tagctccttc ggtcctccga tcgttgctcag aagtaagttg 6300
gccgcagtgt taccactcat gggtatggca gcaactgcata attctcttac tgcctatgcca 6360
tccgtaagat gcttttctgt gactggtgag tactcaacca agtcattctg agaatagttg 6420
atgcggcgac cgagttgctc ttgcccggcg tcaatacggg ataatacgc gccacatagc 6480
agaactttaa aagtgtcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc 6540
ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg cacccaactg atcttcagca 6600
tcttttactt tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa 6660
aaggaataa gggcgacacg gaaatgttga atactcatac tcttcctttt tcaatattat 6720
tgaagcattt atcagggtta ttgtctcatg agcggataca tatttgaatg tatttagaaa 6780
aataaacaaa taggggttcc gcgcacattt ccccgaaaag tgccacctga cgtc 6834

```

&lt;210&gt; SEQ ID NO 6

&lt;211&gt; LENGTH: 6842

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

```

<223> OTHER INFORMATION: The sequence of the CMV expression vector
expressing human codon optimized Vargula luciferase under control
of the CMV promoter

```

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: CMV promoter bases

&lt;222&gt; LOCATION: (209) .. (863)

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: T7 promoter bases

&lt;222&gt; LOCATION: (864) .. (882)

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: Polylinker bases

&lt;222&gt; LOCATION: (889) .. (907)

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: Vargula luciferase gene

&lt;222&gt; LOCATION: (907) .. (6842)

&lt;400&gt; SEQUENCE: 6

```

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtggt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttaggggtag gcgttttgcg ctgcttcgag atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagtccg cgttacataa cttacggtaa atggcccgc tggtgaccg cccaacgacc 360
cccgccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggg aaactgcca cttggcagta catcaagtgt 480
atcatatgcc aagtagccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgcccagta catgacctta tgggactttc ctacttgcca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660

```



-continued

---

actcacgggg	atttccaagt	ctccacccca	ttgacgtcaa	tgggagtttg	ttttggcacc	720
aaaatcaacg	ggactttcca	aatgtcgtg	acaactccgc	cccattgacg	caaatgggcg	780
gtaggcgtgt	acgggtgggag	gtctatataa	gcagagctct	ctggctaact	agagaacca	840
ctgcttactg	gcttatcgaa	attaatacga	ctcactatag	ggagacccaa	gcttgggtacc	900
gagctcatga	agataattat	cctttctgtg	attctggctt	actgtgttac	agtgaattgt	960
caggatgcat	gtccagtaga	ggcggaaaccg	ccatcttcta	ccccgaccgt	accaacctcc	1020
tgcgaagcta	aagaagggga	gtgcatcgat	acaaggtgcg	ctacctgcaa	acgggatatc	1080
ctgtccgacg	gactttgcca	aaataaaccc	gggaagacct	gctgtcgaat	gtgtcagtat	1140
gtcatcgaat	gccgggtcga	ggccgccggg	tattttagaa	cattttacgg	taaacggttt	1200
aatttccagg	aaccggcaa	atacgtactg	gctcgcggca	ccaaggtgg	cgactggagc	1260
gtcacccctga	caatggaaaa	cctggacggg	cagaaaggag	ccgtgcttac	taaaactacc	1320
ctggaggtgg	cgggagacgt	aattgacatc	actcaggcaa	cggctgacct	aataaccgtg	1380
aacggaggag	ctgatcccgt	gattgcaaac	cctttcacta	ttggcgaggt	cacgattgcc	1440
gtcgtcgaaa	ttccaggctt	caacatcaca	gtgatcgagt	tcttcaagct	gatcgtcatt	1500
gatatcctcg	gcgacggtc	cgttcgcac	gcacctgaca	cagccaacaa	gggctgatc	1560
tctggcattt	gtggtaactt	ggaaatgaat	gatgctgatg	acttcacaac	ggacgccgac	1620
caactggcca	ttcaacctaa	tatcaacaaa	gagtttgatg	gatgtccctt	ttacggaaat	1680
ccttcagaca	tcgaatactg	caaaggcctc	atggaaccgt	accgggccgt	ttgcagaaat	1740
aacatcaact	tctactatta	tactctgagc	tgcgcatttg	catactgtat	ggcggtgag	1800
gagagagcca	aacatgtgct	tttcgactat	gtggagacct	gcgccgccc	ggagactcgc	1860
ggtacctgcg	tcctgagcgg	ccataccttc	tatgacacct	tcgataaggc	taggtaccag	1920
ttccaagggc	cttgcaaaga	gctcctgatg	gccgcagatt	gttactggaa	cacttgggac	1980
gtcaaagttt	cccacggga	cgtagagagc	tacacggaag	ttgagaaggt	gaccatcagg	2040
aagcagagta	ccgtcgtaga	cctgatcgtc	gacggcaagc	aggtaaaggt	aggaggcgtg	2100
gacgttagta	ttcgtattc	ttctgaaaat	acgagcatct	actggcagga	tggagacatt	2160
ctgacaaccg	ccatccttcc	agaagctctg	gtggtgaagt	ttaacttcaa	gcagctgctg	2220
gtagtgcaca	ttcgcgacct	attcgacggg	aaaacctgtg	ggatttgcg	caactacaac	2280
caggactcaa	ctgacgattt	ccttgacgcc	gaaggggctt	gcgctcttac	cccaaaccg	2340
cctggatgca	ccgaagagca	aaagcctgaa	gcggaacggc	tgtgcaattc	actgtttgat	2400
tcttcaatag	atgagaaatg	caacgtgtgt	tacaaacctg	accgcatcgc	acgctgcatg	2460
tatgagtatt	gcctgagagg	tcaacaagg	ttctgcgac	acgcgtggga	atttaagaaa	2520
gaatgctaca	taaagcacgg	ggatacattg	gaggtgccgc	cagaatgcca	gtagtctaga	2580
aataattctt	actgtcatgc	caagtaagat	gcttttctgt	gctgcaatag	caggcatgct	2640
ggggatgccc	tgggctctat	ggcttctgag	gcgaaagaa	ccagctgggg	ctctaggggg	2700
tatccccacg	cgccctgtag	cggcgcatta	agcgcggcgg	gtgtggtggt	tacgcgcagc	2760
gtgaccgcta	cacttgccag	cgccctagcg	cccgtcctt	tcgcttctt	cccttcttt	2820
ctcgcacgt	tcgcccgtt	tccccgtcaa	gctctaaatc	ggggcatccc	tttagggttc	2880

-continued

---

cgatthagtg	ctttacggca	cctcgacccc	aaaaaacttg	attaggggtga	tggttcacgt	2940
agtgggccaat	cgccctgata	gacgggtttt	cgccctttga	cgttggagtc	cacgttcttt	3000
aatagtggaac	tcttgttcca	aactggaaca	acactcaacc	ctatctcggc	ctattctttt	3060
gatttataag	ggattttggg	gatttcggcc	tattgggttaa	aaaatgagct	gatttaacaa	3120
aaathtaacg	cgaattaatt	ctgtggaatg	tgtgtcagtt	agggtgtgga	aagtccccag	3180
gctccccagg	caggcagaag	tatgcaaagc	atgcatctca	attagtcagc	aaccagggtg	3240
ggaaagtccc	caggctcccc	agcaggcaga	agtatgcaaa	gcatgcatct	caattagtca	3300
gcaaccatag	tcccgccct	aactccgcc	atcccgc	taactccgc	cagttccgc	3360
cattctccgc	cccatggctg	actaattttt	tttatttatg	cagaggccga	ggccgcctct	3420
gcctctgagc	tattccagaa	gtagtgagga	ggcttttttg	gaggcctagg	cttttgcaaa	3480
aagctcccgg	gagcttgat	atccattttc	ggatctgac	aagagacagg	atgaggatcg	3540
tttcgcatga	ttgaacaaga	tggattgcac	gcaggttctc	cggccgcttg	ggtggagagg	3600
ctattcggct	atgactgggc	acaacagaca	atcggtgct	ctgatgccgc	cgtgttccgg	3660
ctgtcagcgc	aggggcgccc	ggttcttttt	gtcaagaccg	acctgtccgg	tgccctgaat	3720
gaactgcagg	acgaggcagc	gcggtatcg	tggctggcca	cgacgggcgt	tccttgcgca	3780
gctgtgctcg	acgttgtcac	tgaagcggga	agggactggc	tgctattggg	cgaagtgccg	3840
gggcaggatc	tcctgtcatc	tcaccttgc	cctgccgaga	aagtatccat	catggctgat	3900
gcaatgcggc	ggctgcatac	gcttgatccg	gctacctgcc	cattcgacca	ccaagcga	3960
catcgcatcg	agcgagcacg	tactcggatg	gaagccggtc	ttgtcgatca	ggatgatctg	4020
gacgaagagc	atcaggggct	cgcgccagcc	gaactgttcg	ccaggctcaa	ggcgcgatg	4080
cccgcggcg	aggatctcgt	cgtgacccat	ggcgatgcct	gcttgccgaa	tatcatgggtg	4140
gaaaatggcc	gctttctg	attcatcgac	tgtggccggc	tgggtgtggc	ggaccgctat	4200
caggacatag	cgttggctac	ccgtgatatt	gctgaagagc	ttggcggcga	atgggctgac	4260
cgttctcctg	tgctttacgg	tatcgccgct	cccgatcgc	agcgcacgc	cttctatcgc	4320
cttcttgacg	agttctctg	agcgggactc	tggggttcga	aatgaccgac	caagcgacgc	4380
ccaacctgcc	atcacgagat	ttcgattcca	ccgccgctt	ctatgaaagg	ttgggcttcg	4440
gaatcgtttt	ccgggacgcc	ggctggatga	tcctccagcg	cggggatctc	atgctggagt	4500
tcttcgcca	ccccacttg	tttattgcag	cttataatgg	ttacaaataa	agcaatagca	4560
tcacaaattt	cacaaataaa	gcattttttt	cactgcattc	tagttgtggt	ttgtccaaac	4620
tcatcaatgt	atcttatcat	gtctgtatac	cgtcgacctc	tagctagagc	ttggcgtaat	4680
catggtcata	gctgtttcct	gtgtgaaatt	gttatccgct	cacaattcca	cacaacatac	4740
gagccggaag	cataaagtgt	aaagcctggg	gtgcctaata	agtgagctaa	ctcacattaa	4800
ttgcgttgcg	ctcactgcc	gctttccagt	cgggaaacct	gtcgtgccag	ctgcattaat	4860
gaatcgcca	acgcgcgggg	agaggcgggt	tgcgatttgg	gcgctcttcc	gcttctcgc	4920
tcactgactc	gctgcgctcg	gtcgttcggc	tgccgcgagc	ggtatcagct	cactcaaagg	4980
cgtaataacg	gttatccaca	gaatcagggg	ataacgcagg	aaagaacatg	tgagcaaaag	5040
gccagcaaaa	ggccaggaac	cgtaaaaagg	ccgcgttgc	ggcgtttttc	cataggctcc	5100
gccccctga	cgagcatcac	aaaaatcgac	gctcaagtca	gaggtggcga	aaccgcagc	5160
gactataaag	ataccaggcg	tttccccctg	gaagctccct	cgtgcgctct	cctgttccga	5220



-continued

---

```

cctgcecgct taccgatac ctgtccgect ttctcccttc ggggaagegtg gcgctttctc 5280
aatgctcacg ctgtaggtat ctcagttcgg ttaggtcgt tcgctccaag ctgggctgtg 5340
tgcacgaacc ccccgttcag cccgaccgct gcgccttacc cggtaactat cgtcttgagt 5400
ccaaccgggt aagacacgac ttatcgccac tggcagcagc cactggtaac aggattagca 5460
gagcgaggta ttagggcggg gctacagagt tcttgaagtg gtggcctaac tacggctaca 5520
ctagaaggac agtatttggg atctgcgctc tgctgaagcc agttaccttc ggaaaaagag 5580
ttggtagctc ttgatccggc aaacaaacca ccgctggtag cggtggtttt tttgtttgca 5640
agcagcagat tacgcgaga aaaaaaggat ctcaagaaga tcctttgatc ttttctacgg 5700
ggtctgacgc tcagtggaac gaaaactcac gtaagggat tttggtcag agattatcaa 5760
aaaggatctt cacctagatc cttttaaatt aaaaatgaag ttttaaatca atctaaagta 5820
tatatgagta aacttggctt gacagttacc aatgcttaat cagtgaggca cctatctcag 5880
cgatctgtct atttcgttca tccatagttg cctgactccc cgtcgtgtag ataactacga 5940
tacgggaggg cttaccatct ggccccagtg ctgcaatgat accgcgagac ccacgctcac 6000
cggctccaga tttatcagca ataaaccagc cagccggaag ggccgagcgc agaagtggtc 6060
ctgcaacttt atccgctcc atccagtcta ttaattggtg ccgggaagct agagtaagta 6120
gttcgccagt taatagttg cgcaacgttg ttgccattgc tacaggcatc gtgggtgtcac 6180
gctcgtcgtt tggtaggct tcattcagct ccggttccca acgatcaagg cgagttacat 6240
gatccccat gttgtgcaaa aaagcgggta gtccttcgg tctcctcagc gttgtcagaa 6300
gtaagttggc cgcagtgtta tcaactcatg ttatggcagc actgcataat tctcttactg 6360
tcatgccatc cgtaagatgc ttttctgtga ctggtgagta ctcaaccaag tcattctgag 6420
aatagtgtat gcggcgaccg agttgctctt gcccgcgctc aatacgggat aataccgcgc 6480
cacatagcag aactttaaaa gtgctcatca ttggaaaacg ttcttcgggg cgaaaactct 6540
caaggatctt accgctgttg agatccagtt cgatgtaacc cactcgtgca cccaactgat 6600
cttcagcatc ttttactttc accagcgttt ctgggtgagc aaaaacagga aggcaaatg 6660
ccgcaaaaaa ggggaataagg gcgacacgga aatggtgaat actcatactc ttcttttttc 6720
aatattattg aagcatttat cagggttatt gtctcatgag cggatacata tttgaatgta 6780
tttagaaaaa taaacaaata ggggttccgc gcacatttcc ccgaaaagtg ccacctgacg 6840
tc 6842

```

```

<210> SEQ ID NO 7
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Secretory signal

```

```

<400> SEQUENCE: 7

```

```

Met Leu Leu Lys Val Val Phe Ala Ile Gly Cys Ile Val Val Gln Ala
1           5           10           15

```

---

What is claimed is:

1. A multiplexed luciferase assay composition comprising: multiple luciferase reporters, wherein at least two of the luciferase reporters are selected from the group consisting of a firefly luciferase, a *Renilla* luciferase, a *Gaussia* luciferase and a *Cypridina* (*Vargula*) luciferase, and wherein the different luciferase reporters emit at different wavelengths and/or utilize different substrates, and

60 wherein the firefly luciferase is a red-emitting human codon optimized luciferase encoded by SEQ ID NO:3 with an emission maximum of approximately 617 nm; or  
65 wherein the firefly luciferase is a green-emitting human codon optimized luciferase encoded by SEQ ID NO:4 with an emission maximum of approximately 550 nm; or

57

wherein the *Renilla* luciferase comprises A55T, S130A, K136R, A143M, M185V, M253L, and S287L mutations or A123S, D154M, E155G, D162E, I163L, and V185L mutations compared to wildtype *Renilla* luciferase; or wherein the *Vargula* luciferase is encoded by SEQ ID NO: 5 6; and wherein at least one of the luciferase reporters comprises a secretory signal, wherein the secretory signal is peptide sequence of SEQ ID NO: 7.

\* \* \* \* \*

10

58